The cellularisation of mammalian myotubes by myoseverin

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Abstract

Urodele amphibians display a remarkable ability to regenerate and are the only adult vertebrates able to regenerate their limbs. In mammals the capacity for regeneration is greatly curtailed and an important aspect of this is thought to be the plasticity of the differentiated state. On amputation of a newt limb, mature differentiated cell types at the site of injury are able to dedifferentiate to produce a mass of proliferating cells which will eventually re-differentiate to form the tissues of the regenerate. These events are absent in mammals where an inflammatory response predominates.

Skeletal muscle is widely used as a model system for the study of dedifferentiation events in limb regeneration. Skeletal muscle myoblasts withdraw from the cell cycle and fuse to form multinucleate myotubes during differentiation, providing a striking differentiated phenotype. These multinucleate cells undergo conversion to mononucleate cells, referred to as cellularisation, during limb regeneration in urodeles. In recent years, a number of experimental tools to reverse differentiation of mammalian myotubes *in vitro* have been reported, one of which is myoseverin, a small microtubule-binding molecule isolated from a combinatorial chemical library by its ability to induce cellularisation of multinucleate myotubes.

The work in this thesis has further investigated the mechanism of action of myoseverin, assessing to what extent the molecule can be said to initiate a programme in mammalian cells characteristic of that involved in urodele regeneration. Time-lapse microscopy has made possible observation of the process of cellularisation at a single cell level, and of the resulting mononucleate progeny, to determine the fate of these cells following cellularisation, while microarray analysis enabled evaluation of events at a molecular level. These experiments revealed a significant role for microtubules in the mechanism of cellularisation and have highlighted cellularisation as a necessary, but not sufficient, aspect of the complex process of dedifferentiation.

For my family

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Table of contents

List of figures

List of tables

Supporting Data

In addition to the printed thesis, a CD-ROM is included containing supporting data. Please note that this supplementary data is not essential to the arguments contained in this thesis but is intended to complement it, by enabling the viewing of work already represented in the thesis, in an alternative format. The CD-ROM contains movie sequences generated from time-lapse microscopy experiments, and a file containing microarray data. The contents of the CD-ROM is listed below, and you are advised to refer to the relevant page numbers and figures within the thesis for experimental details and comprehensive descriptions.

All files can be read on either a PC or a Macintosh. The file 'Assay_data' is an Excel file and is compatible with both. The time-lapse movie sequences are included in two formats: 'Windows' and 'Quicktime'. PC users are advised to use the files in the 'Windows' folder, these movies will open automatically in Windows Media Player when double-clicked, and are smaller files. For Macintosh users, the files in the 'Quicktime' folder will open in Quicktime.

CONTENTS

from a dual channel time-lapse experiment in which both fluorescence and phase contrast images were captured every 4 minutes. This sequence comprises 300 time points corresponding to 20 hours. The images shown in Figure 3-14 are a detail taken from this slightly larger movie.

Video3 (see Figure 4-8).....121
Purified Pmi28 myotubes were treated with 500nM nocodazole. One image was captured every 3 minutes, the sequence contains 241 images corresponding to 17 hours. Compare the events of this sequence with those of video2.

Video4A (see Figure 5-3A).....166
Purified Pmi28 myotubes were treated with 15μM 109 to induce cellularisation. The resulting mononucleate cell, produced here from a myotube containing three nuclei, is followed for four days subsequently in video4B (see below). Video4A documents the cellularisation event over 24 hours, 287 images were captured at 5 minute intervals.

Video4B (see Figure 5-3B).....167
The mononucleate cell shown to arise during 109 treatment, as shown in video4A, is shown here over a four day period in growth medium. An image was captured every 20 minutes over a 96 hour period, this sequence contains 290 frames. Other mononucleates within the frame can be seen to divide during this time period. Slight fluctuations in brightness and contrast are due to condensation on the lid of the culture dish.

Video5A (see Figure 5-4, A).....169
Purified Pmi28 myotubes were treated with 15μM 109 to induce cellularisation. In this movie, a myotube containing four nuclei undergoes fragmentation to generation two mononucleate progeny, the two cells are followed over a further 4 day period in the subsequent movie (see below). This sequence contains 287 frames, corresponding to almost 24 hours, with one image captured every 5 minutes.

Video5B (see Figure 5-4, B).....169
This sequence shows the events immediately following those depicted in video5A. The cells have been washed to remove 109 and are maintained in growth medium for the

duration of this sequence. One image was captured every 30 minutes, 185 frames are shown here corresponding to 92 hours. Some loss of focus and fluctuations in light are due to the build up of condensation on the lid of the culture dish.

Array_data.....124

This Excel file contains four separate worksheets:

109 – 91 genes altered 1.5-fold upon treatment with 15μM 109 over 24 hours.

NC – 114 genes altered 1.5-fold upon treatment with 500nM nocodazole over 24 hours.

401 – 75 genes altered 1.5-fold upon treatment with 15μM 401 over 24 hours.

Abbreviations – a list of gene names abbreviated in the preceding worksheets.

All fold changes verified as statistically significant are shown in these tables. For 109 and nocodazole, comparisons between 0 and 12 hours, 12 and 24 hours, and 0 and 24 hours were carried out, significant fold changes over each of these three time periods are shown. For 401, comparisons were carried out between 0 and 24 hours only. For details of statistical analysis and gene annotations refer to Materials and Methods.

Abbreviations

BrdU	bromodeoxyuridine
Cdk	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
DM	differentiation medium
DMD	Duchenne muscular dystrophy
DMEM	Dulbeccos modified eagle medium
DMSO	dimethyl sulphoxide
D-PBS	Dulbeccos phosphate buffered saline
ECM	extracellular matrix
eGFP	enhanced green fluorescent protein
EM	electron microscopy
ESC	embryonic stem cell
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocy anate
GM	growth medium
GO	gene ontology
GPI	glycosylphosphatidylinositol
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HSC	haematopoietic stem cell
MEF	myocyte enhancer factor
MHC	myosin heavy chain
MRF	muscle regulatory factor
MTOC	microtubule organising centre
NSC	neural stem cell
NSWB	non-stringent wash buffer
PCM	pericentriolar material
PEC	pigmented epithelial cell
PFA	paraformaldehyde
PIPLC	phosphatidylinositol phospholipase C
RA	retinoic acid

rcf	relative centrifugal force
Rb	retinoblastoma
Silane	3-(trimethoxysilyl)propyl methacrylate
SV40	simian virus 40
SWB	stringent wash buffer
TGF-β	transforming growth factor-beta
TRITC	tetramethyl rhodamine isothiocyanate
UV	ultraviolet

Chapter 1

Introduction

1.1 REGENERATIVE BIOLOGY

1.1.1 An introduction to regeneration

Regeneration is a widespread phenomenon among metazoans with examples in almost every existing phylum, ranging from simple invertebrate organisms to more complex vertebrates and even, to a limited degree, in mammals (Brockes, 1997; Sanchez Alvarado, 2000) (Figure 1-1). One of the most striking examples is the planarian worm. This invertebrate can regenerate its whole body plan from just a small fragment of tissue. Organisms such as starfish and segmented worms possess bi-directional regeneration whereby both fragments of the transected organism can regenerate all missing structures, supporting the hypothesis that regeneration may be linked to asexual reproduction (Sanchez Alvarado, 2000). Among the higher organisms to demonstrate regenerative capabilities are zebrafish, which are used as model organisms for the study of the regeneration of their fin and tail (Akimenko et al., 2003; Poss et al., 2003), tadpole tail regeneration (Slack et al., 2004), deer antler which regenerate annually (Price and Allen, 2004) and, more recently, the MRL mouse, a strain of laboratory mouse which was noticed for its ability to close ear punch wounds and also now appears to demonstrate enhanced cardiac regeneration (Heber-Katz et al., 2004; Leferovich et al., 2001). However, the most impressive example of regeneration among vertebrates lies with the urodele amphibians. The urodele amphibians, which comprise newts and salamanders, can regenerate a remarkable range of tissues and structures (Brockes and Kumar, 2002). The adult newt can regenerate the lens and retina of the eye (Mitashov, 1997; Okada, 1991), lower and upper jaws (Ferretti, 1996; Ghosh et al., 1994), sections of the heart (Bettencourt-Dias et al., 2003; Oberpriller and Oberpriller, 1974), and the tail. It is also the only adult vertebrate capable of regenerating its limbs (Brockes, 1997).

A universal aspect of regeneration is the replacement of missing tissues and structures, and while the mechanisms employed by different animals may differ, a prerequisite for all is a source of cells from which to form the regenerate. Some organisms, such as the freshwater coelenterate *Hydra*, can regenerate in the absence of cell division (Sanchez Alvarado, 2000); other examples, such as lens regeneration in the newt, proceed by proliferation and transdifferentiation of one cell type to another (Okada, 1991). Alternatively, planarian regeneration and limb regeneration in the newt occur via the formation of an intermediate growth zone of poorly differentiated cells called a blastema

(Goss, 1969). Implicit in all examples is an enhanced degree of plasticity in the differentiated tissues of these organisms. Formation of a blastema is the most common mechanism of regeneration and is thought to arise by the dedifferentiation of cells at the site of amputation.

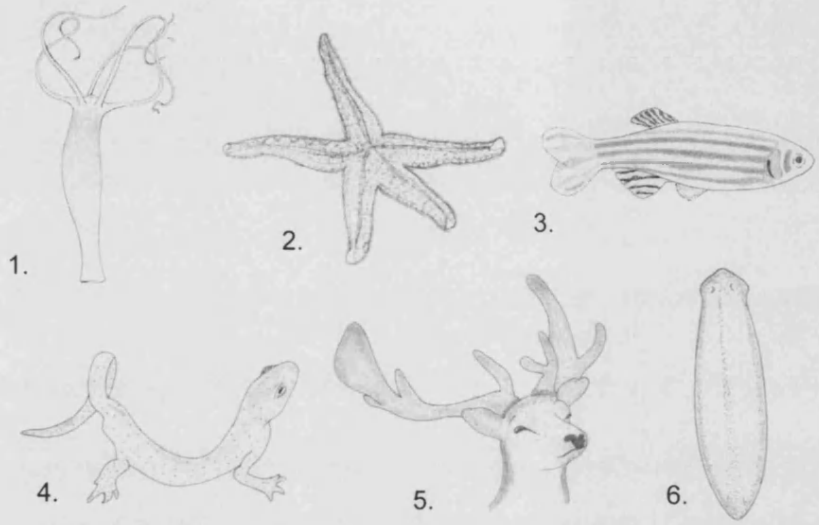


Figure 1-1: Regeneration is a widespread phenomenon among metazoans. A wide range of animals from different phyla possess regenerative abilities, although to differing extents. Hydra (1.), a microscopic freshwater invertebrate, can regenerate a missing head or tail without accompanying cell division. The planarian worm (6.) and starfish (2.) possess bidirectional regeneration whereby a whole organism regenerates from both fragments of the transected organism. The zebrafish (3.) a model organism, is employed to study fin and tail regeneration, and the deer (5.) regenerates its antlers, but the champions of regeneration among vertebrates are the urodele amphibians, which include the newt (4.). The urodeles are the only adult vertebrates able to regenerate their limbs.

An important factor in the ability of urodele amphibians to regenerate, and a possible basis for the disparity in regenerative capacity between urodeles and mammals is thought to be the plasticity of the differentiated state (Brockes and Kumar, 2002). Embryogenesis begins with a single cell that is capable of forming all cell types of the adult organism. As development proceeds, the fate of the cells becomes progressively more restricted and following commitment to a given lineage, cells are no longer capable of contributing to such a wide range of tissues In the adult, terminally differentiated cell types express a tissue-specific phenotype which is considered fixed, and although pools of stem cells do exist in the adult organism, they do not retain the pluripotency of embryonic stem cells and can give rise to only a restricted number of cell types. Earlier experiments had shown, through the use of heterokaryons, that nuclei can be reprogrammed (Blau et al., 1985), and suggested that genetic material was not physically lost during differentiation. The first demonstrations that somatic nuclei, implanted into

enucleated oocytes, could undergo sufficient reprogramming to support embryonic development, were carried out in amphibian cells (Gurdon, 1962a; Gurdon, 1962b; Gurdon and Uehlinger, 1966). In the amphibian system, a number of adult nuclei were shown to give rise to tadpoles following nuclear transplantation into an enucleated oocyte (DiBerardino et al., 1986; Gurdon et al., 1975), and the development of nuclear transplantation technology over subsequent decades, and its application in mammals, ultimately led to a demonstration of the cloning of a whole animal from the nucleus of an adult sheep mammary gland cell injected into an enucleated oocyte (Wilmut et al., 1997). This work established that the genetic information required to form all cell types remains intact in each differentiated adult cell nucleus and that these genes can still be re-activated given the appropriate cytoplasmic environment.

One of the central goals in regeneration research is the identification of the differences between urodeles and mammals which enable newts and salamanders to display such remarkable regenerative capacity while humans possess very little (Tanaka, 2003). The work contained in this thesis centres on the issue of plasticity of the differentiated state in the context of newt limb regeneration, and is concerned specifically with the possibilities for induction of a programme of urodele-like regeneration in a mammalian system. This initial description of certain aspects of regenerative biology will review the events of urodele limb regeneration and the role of dedifferentiation but will also concentrate on how recent advances have provided evidence for plasticity in some terminally differentiated cell types in mammals, and the discovery and potential of tissue specific stem cells. That both differentiated cell types and tissue-specific stem cells may possess an unexpected degree of plasticity highlights the prospects for regenerative biology in mammalian systems. The potential capacity for regeneration in some mammalian cell types has given new impetus to the study of newt regeneration. More than just a fascinating cell biology problem, an understanding of how the differentiated state is regulated in a regenerating organism may eventually be applied to the understanding and manipulation of cell types in mammals.

During regeneration in the urodele, various terminally differentiated cell types at the site of injury are able to contribute to the formation of the replacement tissues or structures, demonstrating a remarkable ability to lose their differentiated characteristics, proliferate,

and then re-differentiate to form the missing structure (Brockes and Kumar, 2002). Limb regeneration requires participation of multiple tissue types at the site of amputation, such as skin, muscle, bone and cartilage, and involves complex pattern formation; regeneration of the limb will be discussed in more detail later in this chapter. In contrast, regeneration of the newt lens is a relatively simple system as it involves the generation of one specific cell type, lens cells, from another, pigmented epithelial cells, and is an excellent example of the plasticity of differentiated cell types seen in the newt.

1.1.2 Transdifferentiation

1.1.2.1 Lens regeneration

Following removal of the newt lens (lentectomy), regeneration occurs from a single tissue, the pigmented epithelial cells (PEC) of the iris. These cells are the only precursors for lens formation. In addition, regeneration occurs only from the dorsal pupillary margin of the iris, never from the ventral cells (Okada, 1991). The initial events of lens regeneration involve the dedifferentiation of PECs which is accompanied by the loss of pigment granules from the cells. This is followed by a period of active proliferation and, after around two weeks, the synthesis of lens-specific crystallin proteins is seen as the cells differentiate to form the tissue of the lens (Goss, 1969; Okada, 1991).

The use of *in vitro* culture methods confirmed that lens tissue could develop from clonal cultures of differentiated PECs, and revealed that chick and mammalian PECs, as well as newt cells, also possessed the ability to transdifferentiate to form 'lentoid' bodies (Eguchi et al., 1974; Eguchi and Okada, 1973). While this cell type switching occurred spontaneously at low frequency after long-term culture, further investigation of culture methods enabled development of conditions in which the dedifferentiation of PECs could be manipulated to give rise to a bipotent cell type capable of forming pigment cells or lens cells (Itoh and Eguchi, 1986). In all cases a period of cell replication preceded re-differentiation into lens cells. These experiments raised the possibility that all vertebrate PECs may possess an inherent ability to transdifferentiate to lens cells.

In culture, the behaviour of cells from the ventral iris was analogous to that of dorsal cells with both able to give rise to lens cells (Eguchi et al., 1974), despite the fact that

only PECs from the dorsal pupillary margin contribute to the regenerate *in vivo*. The reason for this disparity is currently unknown although it has been speculated that cell-cell and cell-substrate interactions may stabilise the differentiated state of PECs *in vivo*, therefore enzymatic disruption of cells in culture may enable ventral PECs to undergo dedifferentiation (Eguchi et al., 1974; Itoh and Eguchi, 1986). More recent work has demonstrated that the protease thrombin is activated selectively at the dorsal margin within minutes of lentectomy (Imokawa and Brockes, 2003; Imokawa et al., 2004). Thrombin has also been implicated in cell cycle re-entry during newt limb regeneration (Tanaka et al., 1999) although its precise role in the events of regeneration remain to be elucidated. The role of transdifferentiation in limb regeneration also remains unclear although examples of the contribution of one lineage in the limb to the tissues of another in the regenerate have been documented. Despite differences in the way missing structures are formed in the newt eye and limb, thrombin may be part of the key to a common mechanism of regeneration.

1.1.2.2 Metaplasia in mammals

In mammals, metaplasia, the switching from one cell type to another, is often associated with pathology, and physiological conditions in which a functional, differentiated cell type is converted into another, are relatively rare, although such examples can be found (Tosh and Slack, 2002). During development of the oesophagus, smooth muscle is gradually replaced by skeletal muscle, the two tissues are distinct at structural, functional and molecular levels. The identification of an intermediate state in which cells transiently express markers of both tissue types suggests that this is a genuine example of transdifferentiation (Kablar et al., 2000; Patapoutian et al., 1995a). In adult mammals, the transdifferentiation of pancreatic cells into hepatocytes in the liver has been documented following certain experimental treatments, and the conversion of chromaffin cells, endocrine cells in the adrenal gland, into neuronal cells has been well studied as a recognised example of transdifferentiation (Okada, 1991). Nonetheless, such plasticity is exceptional and transdifferentiation of terminally differentiated cell types is not a mechanism routinely employed to replace missing or damaged tissue in the mammalian system.

Studying the mechanism by which adult newts are able to replace missing structures is a challenging pursuit, but elucidating the processes involved promises to enhance our

understanding of many aspects of cell cycle control, differentiation and morphology. Regeneration is more than just the study of an abstract phenomenon and at a cellular and molecular level provides information on establishing, maintaining and reversing the differentiated state, enabling identification of the potential for plasticity inherent in our own cells. With the discovery of tissue-specific stem cells and the prospect that many more tissues may harbour them, plus findings that some apparently differentiated cell types retain more plasticity than previously thought, the possibility of manipulating the differentiated state of adult tissues has never seemed more plausible. The use of embryonic stem cells, and more committed cell types, as therapeutics is already in practice and the manipulation or stimulation of host tissues *in vivo* to undergo regenerative events is an attractive possibility for the future of regenerative biology.

1.1.3 Plasticity of the differentiated state in adult mammalian cells

1.1.3.1 Lessons from terminally differentiated cell types

For some tissues, such as epithelium and blood, constant renewal of cells is achieved from a pool of tissue-specific stem cells which divide and differentiate. In other tissues, such as liver and the peripheral nervous system, mechanisms are activated in specific cell types, in response to injury, in order to repair and regenerate the damaged area using differentiated cells. In peripheral nerves, mammalian Schwann cells respond to nerve damage by undergoing de-differentiation and proliferation, followed by re-differentiation during repair, a series of events highly reminiscent of urodele regeneration. Schwann cells respond to axon-derived signals in the peripheral nervous system where they ensheath and myelinate axons; differentiation can be detected biochemically by the expression of myelination proteins and the differentiated Schwann cell also expresses different surface proteins and transcription factors to its undifferentiated counterparts (Scherer and Salzer, 2001). Nerve injury is followed by the degeneration of axons, during this period the myelin sheath is also broken down and the Schwann cells dedifferentiate and re-enter the cell cycle. The endogenous signals which trigger this dedifferentiation event are as yet unknown but an *in vitro* study suggests that the process may be mediated, at least in part, through the activation of the Ras/Raf/ERK pathway (Harrisingh et al., 2004).

The response to damage in the liver is rather more complex as there appears to be a number of cell types which may contribute to regeneration (Dahlke et al., 2004). It has long been known that liver has an intrinsic ability to regenerate and can rapidly restore tissue mass following removal of over 50% of the organ (Michalopoulos and DeFrances, 1997). In the fetal liver, hepatoblasts give rise to two epithelial cell lineages, hepatocytes and biliary cells. In the normal liver there is very little turnover of hepatocytes but following partial hepatectomy, it is this population of cells which rapidly proliferate to replace the lost cells (Fausto and Campbell, 2003). Mature hepatocytes display a terminally differentiated tissue-specific phenotype under normal physiological conditions, but in response to injury they retain the ability to mount a rapid proliferative response. However, despite this efficient mechanism for replacement of lost tissue, the liver has also been found to harbour progenitor cells known as oval cells which are undetectable following partial hepatectomy but can proliferate and differentiate to give rise to hepatocytes and bile duct cells in response to certain types of chemically induced liver damage in which mature hepatocytes are unable to respond efficiently. In addition to oval cells, cell lines have been established from non-hepatocyte epithelial cells isolated from liver and been shown to have a degree of multipotency, and a further population of intrahepatic stem cells known as SHPC (small hepatocyte-like progenitor cells) have been reported to play a role in some types of liver damage (Fausto and Campbell, 2003). Oval cells share some of the markers of haematopoietic stem cells, leading to speculation that liver-derived hepatic stem cells may originate in the bone marrow.

In many cell types, proliferation and differentiation are mutually exclusive fates. Schwann cells and hepatocytes are examples of phenotypically differentiated cell types which display an ability to proliferate and replace damaged or missing tissues, specifically in response to injury. Two further examples of unexpected plasticity in adult mammalian tissues, reported recently, are renin-producing cells (Sequeira Lopez et al., 2004) and pancreatic β-cells (Dor et al., 2004). The case of insulin-producing β-cells illustrates how a differentiated cell type previously believed to arise from stem cells, actually possesses the ability for self-renewal, and that this self-renewal is responsible for the generation of all new β-cells during both normal development and turnover, and under conditions of regeneration. β-cells in the pancreas sense the levels of glucose in

the blood stream and release the hormone insulin, it is these cells which are lost in type I diabetes, so an understanding of how these cells arise is clearly of clinical value.

A number of different stem cells and precursors have previously been implicated in the generation of β-cells but recent work in rodents has provided evidence that in adult life, all new β-cells arise by duplication of existing cells. Using a genetic tagging technique to express a reporter gene only in insulin-producing cells, and activated only following an experimentally administered hormone pulse, investigators stably marked insulin-producing cells present at the time of the hormone pulse and then looked for the presence of the reporter in the pancreas over the course of 12 months. Despite an overall increase in the number of β-cells present, and extensive turnover after 12 months, the percentage of β-cells carrying the tag remained constant. These marked cells must be descendants of the insulin-producing cells in existence at the time of the hormone pulse and demonstrated that duplication of β-cells is the major mechanism by which new β-cells arise. The investigators calculated that any contribution from stem cells or other pancreatic cell types was negligible. In general, differentiated cell types are believed to arise, ultimately from pluripotent stem cells, via progressively more restricted precursors. The work described above shows that in some cases terminally differentiated cells can arise directly by the duplication of pre-existing terminally differentiated cells.

A similar conclusion although under somewhat different circumstances can also be drawn from recent work revealing the origin and fate of renin cells (Sequeira Lopez et al., 2004). Renin-producing cells play an essential role in the homeostasis of blood pressure and of the volume and composition of the extracellular fluid. In adult animals they are located in the nephron, in the wall of the afferent arteriole next to the glomerulus where they can respond to changes in blood pressure and electrolyte absorption. Renin hydrolyses angiotensin I which is then cleaved to produce angiotensin II, a potent vasoconstrictor. The effects of angiotensin II lead to changes in heart rate, blood pressure and the resorption of water and sodium chloride. In the adult, renin production is limited to just a few specialised cells with a restricted location in the nephron but during embryogenesis, renin-producing cells are broadly distributed throughout the glomeruli and the intrarenal arteries. Although hormone production is considered a differentiated phenotype these embryonic renin-producing cells are precursors for

multiple cell types and as renin production becomes restricted, many of these cells differentiate into smooth muscle cells of the renal arterial network. However, even at this stage it appears that the differentiated state of these former renin-producing is not irreversibly fixed; in the adult, under conditions of extreme stress such as dehydration or hypotension, these cells can switch back to their renin-producing phenotype. Here is an example of a terminally differentiated cell type which retains a degree of plasticity that is based on its developmental history. As for Schwann cells and liver cells, these phenotypic changes are activated in response to trauma suggesting that such examples of plasticity exist to enable the body to respond to stress.

The cell types described in this section collectively demonstrate how our understanding of differentiation, the origin of differentiated cell types and their in-built capabilities is expanding. By looking beyond long-held beliefs on the nature of the differentiated state, scientists are increasingly able to reveal the inherent potential, previously unsuspected, in adult differentiated cell types.

1.1.3.2 Tissue-specific stem cells

While some tissues, such as liver and peripheral nerves described above, posses the ability to regenerate, by activating differentiated cell types, in constantly regenerating tissues like epithelium and blood a different mechanism must exist to maintain the integrity of the system. In these cases a resident pool of undifferentiated stem cells exists to constantly renew the tissue. Although tissue-specific stem cells do not undergo dedifferentiation prior to activation, as they are, by definition, less restricted than their differentiated counterparts, an understanding of regenerative events will have significant impact on this field by allowing investigators to understand and direct the differentiated state of stem cells. Increasingly it appears that in the case of tissue-specific stem cells too, as in some of the cases of adult differentiated cell types described above, we may not fully appreciate the potential of these cells.

The term 'tissue-specific' implies a degree of commitment to a specific lineage. Satellite cells are a well-studied example of one such lineage-committed cell type. They arise in skeletal muscle during myogenesis and reside alongside the plasma membrane of myofibres under the basal lamina in a quiescent state unless activated by muscle growth

or damage. Satellite cells possess the properties of proliferation to give rise to myogenic progenitor cells, and self-renewal, and they are responsible for the increase in muscle mass during post-natal muscle growth (Schultz, 1989). Mice that do not develop satellite cells are small and weak, and demonstrate reduced muscle fibre size and mass, indicative of a deficient period of post-natal growth (Seale et al., 2000). Satellite cells are also activated during muscle regeneration in response to injury (Schultz, 1989; Seale and Rudnicki, 2000) although the exact combination of growth factors and signalling molecules responsible for *in vivo* activation of satellite cells remains elusive.

There is evidence from studies in mice that there exists in skeletal muscle an additional population of muscle-specific stem cells. These cells, termed the Side Population (SP) have been isolated from skeletal muscle by cell sorting methods based on the expression of haematopoietic stem cell markers such as Sca-1 and c-kit on the surface of these cells (Gussoni et al., 1999; Jackson et al., 1999; Lee et al., 2000b). When injected into the bone marrow of lethally irradiated mice, SP cells were able to rescue the mice and reconstitute all lineages of the haematopoietic system (Gussoni et al., 1999; Jackson et al., 1999) as well as contributing to myofibres in skeletal muscle and enhancing regeneration when injected into the muscle of dystrophin-deficient, mdx mice (Gussoni et al., 1999; Lee et al., 2000b). SP cells clearly represent a cell population distinct from satellite cells as demonstrated by their persistence in Pax7 mutant mice which lack satellite cells (Seale et al., 2000). They may possess the potential to give rise to satellite cells (Asakura et al., 2002; Gussoni et al., 1999), although it has been suggested that the population of cells may not truly be muscle-derived and could instead originate in the haematopoietic system (McKinney-Freeman et al., 2002).

In addition to a muscle-derived stem cell population capable of undergoing haematopoietic differentiation, the opposite has also been demonstrated with labelled haematopoietic stem cells (HSC). HSC reside in the bone marrow and give rise to all major blood cell lineages. The blood system is one which is constantly regenerating in order to maintain itself and so differs from the earlier examples of skeletal muscle and liver in which stem cell populations are activated specifically in response to stress or injury. Following injection into bone marrow or into muscle at the site of injury, HSC were later found incorporated into skeletal muscle myofibres (Ferrari et al., 1998;

Gussoni et al., 1999) raising the possibility that cells from the bone marrow may be recruited to sites of injury to enhance the regeneration of muscle.

Similar results were found in cases of liver damage. Female rats were lethally irradiated and then rescued by injection with bone marrow from a male donor. Following experimentally induced liver damage to activate regeneration, both hepatocytes and oval cells containing the Y chromosome were identified (Petersen et al., 1999). Examination of human patients who received cross-gender whole liver or bone marrow transplants also revealed the presence of bone marrow derived cells in liver epithelium and supported the hypothesis for an extra-hepatic source of cells which may be mobilised under conditions of regeneration (Alison et al., 2000). The actual mechanism by which bone marrow derived cells contribute to liver regeneration remains obscure however, and in some models of liver damage at least, fusion with hepatocytes rather than transdifferentiation of bone marrow cells is credited with the potential to enhance regeneration (Dahlke et al., 2004).

The central nervous system has been thought to demonstrate very little regenerative capacity so the discovery of neural stem cells (NSC) announced the possibility for increased plasticity in the system. NSC were thought to give rise only to neural tissues and to only three cell types, astrocytes, oligodendrocytes and neurons but when labelled adult or embryonic NSC were injected into sub-lethally irradiated Balb/c mice, they were found to contribute to the haematopoietic system (Bjornson et al., 1999). Up to 12 months after injection, labelled cells were found in the spleen and bone marrow and when isolated from the bone marrow were found to express markers not expressed by the original NSC, and form all lineages of the haematopoietic system *in vitro*.

These experiments have challenged the view that adult stem cells are only able to give rise to cell types of their tissue of origin and have demonstrated a remarkable degree of plasticity in the adult mammalian system. So-called tissue-specific stem cells from muscle, liver, central nervous system and even the dermis (Toma et al., 2001) have been shown to possess pluripotency beyond that expected of them and it seems likely that similar cell populations exist in many adult tissues. However, while these reports may seem to suggest that recruitment of tissue-specific stem cells from various parts of the

body to sites of injury may be a viable therapeutic approach in the future, some caution must be exercised in reaching the conclusion that such stem cell populations hold the key to mammalian tissue regeneration. In many of the experiments described above in which cell populations were injected or implanted, the contribution of tissue-specific stem cells to other tissues was small and may or may not occur under physiological conditions. In addition, it has been suggested that cases of apparent transdifferentiation by tissue-specific stem cells are in fact due to fusion of the transplanted cells with the resident population (Ying et al., 2002). This appears to be the case where injection of haematopoietic stem cells rescued mice with a genetic liver disease, delivery of the vital missing enzyme to the liver was shown to result from fusion with the host hepatocytes rather than transdifferentiation (Vassilopoulos et al., 2003; Wang et al., 2003). Nonetheless, the results described here show that a wide range of adult stem cells may possess much greater plasticity than previously thought, and determining the extent of their pluripotency and the signals to which they respond, may indeed prove a valuable tool in regenerative medicine and research.

1.1.4 Plasticity of the differentiated state during urodele limb regeneration

So far, this chapter has largely concentrated on the capacity of mammalian cells in order to demonstrate that regeneration research, far from being an obscure phenomenon in urodele amphibians, possesses real potential for other systems and for human therapeutic approaches. That terminally differentiated cell types in the mammalian system are continuing to reveal a previously unknown, albeit relatively limited, degree of plasticity, provides optimism that a greater understanding of the molecular mechanisms of regeneration, gleaned from study of the urodeles, may ultimately enable us to induce regeneration-like processes in mammals.

Regeneration of the newt limb involves plasticity of the differentiated state of cells in multiple tissues including epithelium, muscle, cartilage and bone, some of which have been discussed above in the context of mammalian plasticity. Limb regeneration provides an excellent system in which to study the cellular processes of dedifferentiation in these cell types, and the mechanism by which tissue damage can stimulate the activation of terminally differentiated cell types, resulting in regeneration of a complex structure.

1.1.4.1 The events of urodele limb regeneration

Following amputation of the urodele limb, one of the first events to take place is the covering of the wound surface with a specialised epithelium. Within hours, the epithelial cells at the amputation site are mobilised and migrate to cover the exposed surface, this process occurs in the absence of DNA synthesis or mitosis (Tsonis, 1996). The resulting wound epidermis is distinct from the thicker epidermal layer of normal skin and lacks underlying dermal cells and specialised glands. The wound epithelium has been shown to be essential for the subsequent outgrowth of the regenerating limb. When it is removed or its formation is prevented, cells at the amputation site appear to undergo some early dedifferentiation events and limited proliferation but without the instructive influence of the overlying epidermis, the cells soon differentiate and no regeneration occurs (Mescher, 1976).

Around four days after amputation, a mass of proliferating mesenchymal cells begins to form beneath the epithelium, at the tip of the stump, although the timing of these events may depend on the specific species of urodele amphibian and on the age and size of the animal. These cells arise by the dedifferentiation of the cell types at the site of amputation including muscle and cartilage, and form a cone of proliferating mononucleate cells known as the blastema (Goss, 1969; Tsonis, 1996). The cells of the blastema appear morphologically identical although it is likely that at a molecular level, distinct subpopulations may exist. The cells are mesenchymal, but otherwise have lost the specialised characteristics of their tissue of origin.

As the proliferation of mononucleate blastemal cells generates outgrowth of the regenerate, redifferentiation to form the new tissues of the replacement limb begins in a proximal to distal direction. At the tip of the regenerate a pool of cells are maintained in a proliferative and undifferentiated state by signals from the overlying epidermis, in a manner analogous to the instructions from the AER (apical ectodermal ridge) to the underlying progress zone during chick limb bud development. Only the missing structures are regenerated so a wrist blastema gives rise to a hand while a shoulder blastema regenerates the entire limb. The regenerate contains all the cell types and tissue organisation of the original limb (Goss, 1969). The entire regeneration process takes approximately four weeks.

1.1.4.2 Origin of the blastema

An important issue surrounding limb regeneration has been to establish the origin of the cells that form the proliferating mass of the blastema and the relative contribution of the different tissues present in the stump. Early debates were based around the controversy that limb regeneration in the urodele amphibians arose by dedifferentiation of mature tissues rather than by the activation of resident populations of stem cells, such as satellite cells, which were known to be the source of cells for muscle regeneration in mammals. However, electron microscope observations (Hay, 1959; Hay and Fischman, 1961) and transplantation of labelled cells and tissues (Tsonis, 1996) provided evidence for dedifferentiation as a major event in limb regeneration and subsequently many studies established the contribution of different tissues to the blastema and the resulting regenerate.

The isolation of monoclonal antibodies identifying cells and tissues of the limb and blastema further supported the model of dedifferentiation. Antibody 22/18 recognised an antigen expressed exclusively on cells of the blastema, being undetectable in the tissues of the normal limb (Kintner and Brockes, 1984), and was proposed to identify a myogenic subpopulation within regenerating newt limb (Griffin et al., 1987). At early stages of blastema formation, the 22/18 antibody was found to colocalise, on a small proportion of cells, with markers for either muscle fibres or Schwann cells, suggesting that acquisition of the 22/18 antigen was indicative of the transition from terminally differentiated cell types to blastemal cells (Kintner and Brockes, 1985). The antibody was later found to specifically recognise an intermediate filament component (Ferretti and Brockes, 1990). Using a variety of cellular markers, muscle (Griffin et al., 1987; Kintner and Brockes, 1984), dermis (Muneoka et al., 1986), cartilage (Casimir et al., 1988) and Schwann cells (Kintner and Brockes, 1985) have all been shown to make a contribution to the blastema, while epidermal cells do not, giving rise only to the epidermis of the regenerate (Hay and Fischman, 1961).

The capacity for metaplasia during limb regeneration has also been investigated at length. While lens regeneration in the newt provides a clear example of transdifferentiation from the pigmented epithelial cells of the iris, into lens cells, the extent to which such processes occur in the limb is unclear. Whether the dedifferentiation of mature tissues of

the stump proceeds to a degree where the mononucleate blastemal cells are bipotent or even multipotent, or whether the cells are ultimately capable only of re-differentiating into cells of their tissue of origin is an interesting aspect of the plasticity of differentiated cells. Several studies have identified cases of metaplasia under the conditions of limb regeneration. Experiments in which the humerus of the intact limb was removed, followed by amputation at the elbow, strongly implied that metaplasia occurred during regeneration. Despite the absence of bone in the stump, skeletal elements developed normally in the regenerate distal to the site of regeneration suggesting that the cells which contributed to the regenerate could be derived by metaplasia of other cell types; the existence of markers from muscle and connective tissue in the newly formed cartilage supported this (Casimir et al., 1988). Implantation of labelled muscle cells into limb blastemas also reportedly gave rise to small numbers of labelled cartilage cells in the regenerate (Kumar et al., 2000; Lo et al., 1993).

It has been argued that reserve stem cells in the limb are a source of cells for the regenerate. The discovery of a myogenic cell population capable of proliferation in urodele muscle, termed the post-satellite cell, supported this view (Cameron et al., 1986; Popiela, 1976). While a role for such stem cells has not been entirely ruled out, neither has it been convincingly demonstrated, and although a contribution by these cells may take place, the evidence for dedifferentiation as the major source of blastemal cells has continued to mount. From early electron microscopy studies, increasingly sophisticated techniques have enabled the tracking of cells within the blastema and observation of myofibres *in vivo* allowing investigators to make increasingly comprehensive observations of the cellular events of regeneration. The sum of such experiments now makes it difficult to dispute the occurrence of dedifferentiation of mature cells following amputation in the newt, and the subsequent contribution of these cells to growth of the blastema and ultimately formation of a new limb. Some of the experiments, providing progressively more compelling evidence for these events, are reviewed later in this chapter, where the mechanism of dedifferentiation is dealt with in more detail.

That the blastema provides an environment permissive to the destabilisation of the differentiated state was demonstrated convincingly by Reyer, through the implantation of iris cells at ectopic sites in the newt. As described earlier, it is the PECs (pigmented

epithelial cells) from the dorsal margin of the iris, which demonstrate plasticity of their differentiated state in response to lentectomy, undergoing dedifferentiation and proliferation followed by redifferentiation to form the cells of the missing lens (Okada, 1991). When dorsal iris was implanted into the blastema of a regenerating newt limb some remarkable observations were made. In the environment of the blastema, PECs of dorsal iris tissue lost pigmentation and underwent transdifferentiation, with 64-76% of iris implants forming a lens in the limb blastema (Reyer et al., 1973). Iris implantation was performed at alternative sites including the dorsal fin, brain and shoulder but the frequency and efficiency of lens formation in the blastema could not be replicated elsewhere. These results show that the blastema represents a unique environment in which the differentiated state is destabilised, providing the appropriate cellular and molecular signals for dedifferentiation and proliferation.

In order to efficiently study the processes of dedifferentiation during urodele limb regeneration, and assess the findings in relation to other organisms, a relevant cell culture system is essential. Skeletal muscle is a major tissue of the limb and the availability of myogenic cell lines, plus the amenability of muscle explants to culture *in vitro*, has made it an often used model system for such studies. The work contained in this thesis describes the use of mammalian skeletal muscle cell lines to investigate the possible mechanism of dedifferentiation, which appears to be a central process in urodele regeneration, and to assess the potential of the mammalian system to undergo such events. In order to understand the process of dedifferentiation and so evaluate the plasticity of the differentiated state, it is vital to first have knowledge of the events and molecules involved in the acquisition of the differentiated phenotype. It has been suggested that regeneration, rather than activating a specialised battery of genes, occurs as a recapitulation of the differentiation process which takes place during development. If this is the case, then an understanding of muscle differentiation and development is fundamental to the research of regeneration (Zhao and Hoffman, 2004).

1.2 MYOGENESIS

Terminally differentiated muscle comprises multinucleate, post-mitotic myotubes. During differentiation, committed myogenic myoblasts withdraw from the cell cycle and

fuse to form multinucleate myofibres with accompanying expression of muscle-specific markers. The transition from a mononucleate to a multinucleate state, and withdrawal from the cell cycle provide two indices of differentiation, which enable the process to be followed and quantitated making skeletal muscle an ideal system in which to study the events of differentiation. Equally, the properties of the system are then suited for the detection of dedifferentiation events in the context of regeneration. As mentioned earlier, skeletal muscle is also a physiologically relevant system in terms of events occurring in the limb and is a well characterised model system for investigating the events of limb regeneration.

Myogenic differentiation is a complex process and proceeds by a carefully regulated series of consecutive events. The cells undergo commitment, proliferation, withdrawal from the cell cycle and finally fusion to form post-mitotic multinucleate myotubes.

1.2.1 Commitment to the myogenic lineage

The myogenic regulatory factors (MRFs) comprise a group of related basic helix-loop-helix (bHLH) transcription factors which coordinate the determination and differentiation of the myogenic lineage. Also referred to as the MyoD family, after the first member to be identified, the four muscle regulatory factors, MyoD, Myf-5, myogenin and MRF4, have all been shown to induce myogenic differentiation when expressed in non-muscle cell types (Olson, 1990; Weintraub et al., 1991).

During development, the four genes display distinct spatial and temporal patterns of expression. All skeletal muscle of the body originates from the somites. Somites are transitory structures which arise during embryogenesis as blocks of epithelium on either side of the neural tube. The cells of newly formed somites are multi-potent but gradually become committed to forming one of a number of different cell types, including cartilage, dermis, connective tissue and muscle, under the influence of signals from surrounding tissues such as the notochord and neural tube (Buckingham, 2001; Zhao and Hoffman, 2004) . The region of the somite termed the dermomyotome gives rise to skeletal muscle. Myf-5 is the first of the MRFs to be expressed in the somites and in the

limb buds (Ott et al., 1991), myogenin is activated shortly after, followed by MRF4. MyoD is the last of the four to be expressed, around two days after Myf-5 is detected (Sabourin and Rudnicki, 2000). Myf-5 and MRF4 are expressed only transiently; Myf-5 is expressed during one specific period in early development and is present only at low levels in adult muscle. MRF4 shows a biphasic pattern of expression, expressed first in the somites, following myogenin induction, but then declining as the myotomes develop, its expression is re-activated several days later as skeletal muscle begins to differentiate in the foetus (Bober et al., 1991). In contrast, expression of myogenin and MyoD is maintained once activated.

Another gene with a significant role in muscle development is Pax3. Pax3 is a member of a family of paired-box-containing transcription factors with roles in regulating developmental process. It is expressed initially throughout the newly formed somites but becomes restricted to the dermomyotome (Daston et al., 1996). A subpopulation of myogenic precursors in the somite will form the skeletal muscle of the limbs, these cells must migrate away from the somite into the limb bud. Expression of Pax3 and c-met, a tyrosine kinase receptor, on these cells is required for migration. In *Splotch* mice, which contain a mutation in Pax3, the phenotype is embryonic lethal and the animals fail to develop any limb muscles, this has been shown to be due to a defect in migration rather than a lack of specified muscle progenitors (Daston et al., 1996). During migration to the limb bud, the cells are maintained in a proliferating and undifferentiated state and do not express MRFs until they reach the site of myogenesis in the limb (Birchmeier and Brohmann, 2000; Rawls and Olson, 1997). Pax3 has been shown to induce MyoD and activate myogenesis in non-muscle cells, just as each of the MRFs can (Maroto et al., 1997), but the specific role of Pax3 in myogenesis remains unclear (Borycki and Emerson, 1997). It is thought that the co-expression of Pax3 with the homeobox gene, Msx-1, a transcriptional repressor, during migration may prevent the induction of MRFs and maintain the cells in an undifferentiated state (Bendall et al., 1999), while the expression of Pax3 is sufficient to maintain the cells in the muscle lineage despite the absence of MRFs (Rawls and Olson, 1997). Expression of relevant transcription factors during limb muscle migration, commitment and differentiation is shown in Figure 1-2.

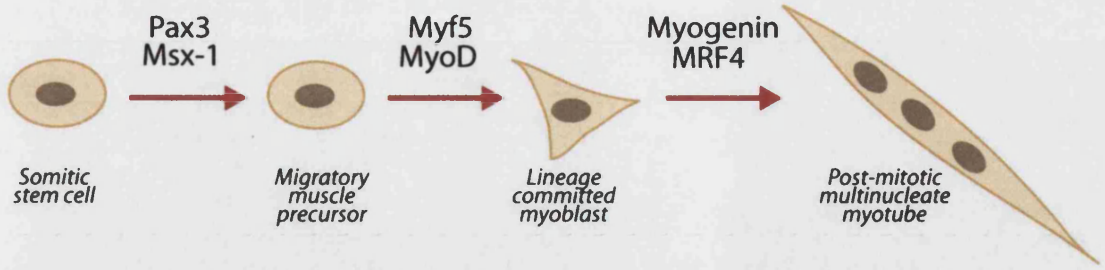


Figure 1-2: Schematic of limb muscle development. All skeletal muscle of the body derives from stem cells in the somites. Cells which will form the skeletal muscle of the limb must migrate from the somite to the limb bud, these migratory cells express Pax3, a marker of early muscle precursors, and Msx-1 which maintains the cells in an undifferentiated state during migration. On arrival at the site of myogenic differentiation, induction of the muscle regulatory proteins Myf-5 and MyoD mediate cell commitment to the myogenic lineage, later, expression of myogenin and MRF4 controls differentiation of myoblasts to form post-mitotic, multinucleate myotubes.

Generation of knock-out mice for each of the MRFs, individually and in combination, has enabled classification the MRFs into two groups, involved in the specification or differentiation of muscle progenitors. Myf-5 and MyoD regulate the specification of the myogenic lineage and all somite-derived muscle cells express these two factors (Sassoon et al., 1989; Tajbakhsh and Buckingham, 2000). Myogenin and MRF4 do not appear to be involved in these early events of commitment to the muscle lineage but are required for differentiation into mature muscle fibres. The study of mice carrying null mutations for each of the MRFs has also demonstrated some degree of redundancy among the four genes.

MyoD knockout mice were born healthy and fertile with no apparent defects. Histology, immunohistochemistry and ultrastructural studies detected no difference between the musculature of MyoD-/- and wild type mice. Of significance however, was an observed 3.5-fold increase in expression of Myf-5, resulting in high levels of expression even at times when Myf-5 levels are low during normal development, suggesting that Myf-5 is able to compensate for the loss of MyoD (Rudnicki et al., 1992).

The Myf-5 null mice also showed essentially normal skeletal muscle and were of normal size and weight but died within minutes of birth due to respiratory failure caused by a rib defect (Braun et al., 1992). The normal skeletal muscle of these animals suggests that other MRFs are capable of maintaining the myogenic programme in the absence of Myf-5, although development of some muscles was delayed, but it appears that Myf-5 plays a

unique role in the development of a complete rib cage which cannot be compensated for by other factors.

Earlier tissue culture studies had presumed that the MRFs would play a vital role in muscle development, and the finding that both MyoD and Myf-5 knockout mice were able to develop grossly normal musculature challenged the view that the MRFs each regulated distinct aspects of myogenesis. The essential role of MyoD and Myf-5 as factors which regulate the early events of determination of the myogenic lineage, was confirmed, however, by examination of double knockout mice lacking both genes. These mice died very soon after birth and showed a striking absence of skeletal muscle. Other organs developed apparently normally but no myoblasts were detected, and closer examination revealed no expression of myogenin or MRF4 and a complete absence of muscle-specific proteins such as MHC, troponin T and α-actin (Rudnicki et al., 1993).

Myogenin is not involved in the early determination of muscle but is required for differentiation of myotubes as revealed by targeted disruption of the myogenin gene. The knockout mice died immediately after birth and showed no signs of movement. Closer investigation showed a dramatic reduction in muscle mass and in some regions, such as the limb, containing muscle fibres in wild type mice were populated by mononucleate cells in the myogenin knockout (Hasty et al., 1993; Nabeshima et al., 1993). This suggested that myoblasts were specified and correctly positioned but had failed to fuse to form multinucleate fibres. The mice also displayed rib defects. The MRF4 knockout mouse also showed incomplete rib development and died of respiratory failure but in contrast to the myogenin knockout had largely normal skeletal muscle, possibly due to an observed up-regulation of myogenin, which may compensate for loss of MRF4 (Patapoutian et al., 1995b).

The interactions between the four MRFs are complex (Molkentin and Olson, 1996). Although MyoD and Myf-5 appear to compensate for one another, their different expression patters during embryogenesis, and subtle defects in the knockout mice suggest that they do have distinct roles in myogenesis (Kablar et al., 1997; Sassoon et al., 1989). Insertion of the myogenin gene at the Myf-5 locus rescues the rib defect seen in Myf-5 null mice (Wang et al., 1996), but MyoD over-expression cannot compensate for

loss of myogenin in cultured cells (Myer et al., 2001). While Myf-5 is the first of the MRFs to be expressed in the embryo, it cannot support myogenesis in compound mutants lacking functional copies of the other MRF genes (Valdez et al., 2000). However, collectively the results have led to the classification of MyoD and Myf-5 as the master myogenic determination genes while myogenin and MRF4 are required for later steps in differentiation.

While *in vivo* studies provided valuable information on the roles of each of the MRFs in embryonic development, the use of cultured myogenic cell lines provides a more convenient and tractable system and has enabled further elucidation of the molecular aspects of MRFs, their binding partners and interactions with other proteins. The MRFs are muscle-specific transcription factors and interact with a variety of other tissue-specific and general transcription factors to regulate myogenesis (Puri and Sartorelli, 2000). The E proteins, such as E12 and E47 (products of the E2A gene), are ubiquitous bHLH proteins and heterodimerise with the MRFs to produce active complexes. A second group of transcription factors, the MEF2 family are expressed in cardiac, skeletal and smooth muscle during development and have also been shown to activate muscle-specific transcripts (Neuhaus and Braun, 2002). MEF2 proteins interact directly with the MRF/E protein heterodimers, and the two families of muscle-specific transcription factors work in concert to promote myogenic differentiation, regulating one anothers expression, along with other muscle-specific genes (Naidu et al., 1995; Naya and Olson, 1999).

Among the proteins which act as negative regulators of the MRFs are the Id proteins. This family of four HLH proteins all lack a DNA-binding domain and are induced by serum thus inhibiting differentiation under growth conditions. Most HLH factors function through the formation of heterodimers, which enables Id proteins to bind to, and act as dominant negative inhibitors of, other HLH proteins including the myogenic bHLH transcription factors and their binding partners, the ubiquitous E proteins (Puri and Sartorelli, 2000; Sabourin and Rudnicki, 2000). While Id protein over-expression is able to delay differentiation it is not sufficient to completely inhibit it suggesting that there are other mechanisms that inhibit the MRFs under growth conditions.

In myogenic cell lines in culture, most proliferating myoblasts express MyoD and Myf-5, although they appear to be oppositely regulated during the cell cycle with MyoD peaking during G1 at a time when Myf-5 is low (Buckingham, 2001; Kitzmann and Fernandez, 2001). During myogenic differentiation the cells continue to express MyoD, while Myf-5 decreases (Dedieu et al., 2002; Shimokawa et al., 1998). Myogenin is expressed upon commitment to myogenic differentiation, prior to cell cycle withdrawal and fusion (Andres and Walsh, 1996; Walsh and Perlman, 1997), and increases during early stages of differentiation (Dedieu et al., 2002; Shimokawa et al., 1998) while MRF4 is associated with maturation of muscle fibres and was not detected in myotubes of the C2C12 myogenic cell line (Dedieu et al., 2002). Such expression patterns are consistent with the conclusions from knockout mice that MyoD and Myf-5 are involved in the early determination of myoblasts while myogenin and MRF4 are activated later, and are associated with differentiation. In culture, myoblasts continue to proliferate in the presence of serum but can be induced to undergo myogenic differentiation to form post-mitotic, multinucleate myotubes by lowering the serum concentration (Florini et al., 1991; Olson, 1992), enabling the study of critical aspects of myogenic differentiation, such as cell cycle withdrawal and fusion, *in vitro*.

1.2.2 Cell cycle withdrawal

Following commitment to the muscle lineage, cells undergoing differentiation withdraw from the cell cycle and fuse to form post-mitotic multinucleate myotubes (Walsh and Perlman, 1997). Exit from the cell cycle is an early event in myogenic differentiation that precedes fusion and the expression of late muscle markers such as myosin heavy chain (MHC). Muscle differentiation is coordinated with the cell cycle through the interaction of MRFs and cell cycle regulatory proteins (Molkentin and Olson, 1996). Before describing these interactions, there follows an overview of cell cycle regulation with emphasis on the G1-S transition, as cell cycle withdrawal occurs in G1 and maintenance of the post-mitotic state involves the regulation of key proteins at this crucial checkpoint. This summary is not intended to provide an exhaustive review of the cell cycle but instead will concentrate on the aspects of cell cycle regulation that are relevant to myogenesis.

1.2.2.1 Overview of the G1/S transition in mammalian cells

Cyclin-dependent kinases (Cdks) are a highly conserved family of proteins which play a central role in regulating all the major cell cycle transitions in mammalian cells. They consist of a catalytic Cdk subunit, and a regulatory cyclin component as cyclin binding is required for kinase activity. During G1, two classes of Cdks are important; the D-type cyclins (of which there are three) complexed with either Cdk4 or Cdk6, and cyclin E which complexes with Cdk2 (Ekholm and Reed, 2000). Cyclin D-Cdk4/6 and cyclin E-Cdk2 phosphorylate different target proteins and play distinct roles in progression of the cell cycle.

Cdk activity is regulated at a number of levels; by phosphorylation, the availability of cyclins, and by cyclin-dependent kinase inhibitors (CKIs). Cyclin D accumulates during early G1 while cyclin E is active much later in G1 (Ekholm and Reed, 2000), which is consistent with cyclin E being downstream of cyclin D (Geng et al., 1999). Cyclin D is the major mitogen-responsive cyclin of the cell and is thought to act as a growth factor sensor, providing a vital link between mitogenic cues and the cell cycle machinery (Sherr and Roberts, 1999). In the absence of cyclin D cells arrest in G1 (Lukas et al., 1994), highlighting the significance of cyclin D-dependent kinase activity in driving cell cycle progression.

Cyclin D-Cdk4/6 acts to promote cell cycle progression in two ways; by directly phosphorylating the product of the retinoblastoma gene (Rb) and by sequestering CKIs. Rb is a tumour suppressor gene, first identified as mutated in cases of retinoblastoma, a retinal tumour, and as such is a key regulator of the cell cycle (Hatakeyama and Weinberg, 1995). Among the proteins bound by Rb are the E2F family of transcription factors (Mulligan and Jacks, 1998; Nevins, 1992). E2F induces the transcription of a panel of genes required for cell cycle progression including cyclin E and components of the DNA synthesis machinery. When bound and sequestered by Rb, E2F is prevented from activating transcription and there is evidence that the Rb-E2F protein complex actively represses transcription of certain genes (Harbour et al., 1999; Sherr and Roberts, 1999; Weintraub et al., 1995). In order to bind E2F, Rb must be in its active hypophosphorylated form. Phosphorylation of Rb by Cdks releases E2F and drives the cell through G1 into S phase.

Both cyclin D-Cdk4/6 and cyclin E-Cdk2 are required for full hyperphosphorylation of Rb (Harbour et al., 1999; Sherr and Roberts, 1999), and prior phosphorylation of the protein by cyclin D-Cdk4/6 is required for the subsequent phosphorylation by cyclin E-Cdk2. The initial phosphorylation by cyclin D-Cdk4/6 is thought to bring about a conformational change which blocks active repression of gene transcription by the Rb-E2F protein complex but is not sufficient to disrupt the complex and release active E2F; this function is carried out by cyclin E-Cdk2 which is now able to complete the phosphorylation of Rb (Harbour et al., 1999). Hyperphosphorylation of Rb leaves E2F free to induce transcription of its target genes, one of which is cyclin E, thus establishing a positive feedback loop to drive the cell cycle forward. Cyclin E may also be a target of the active repression complex, explaining in part why prior phosphorylation of Rb by cyclin D-Cdk4/6 is required for Cdk2 activity (Harbour et al., 1999).

1.2.2.2 Cyclin-dependent kinase inhibitors

In addition to Rb phosphorylation, a second major role for cyclin D-Cdk4/6 in regulating the G1/S transition is binding and sequestering of CKIs. There are two classes of CKIs, the INK4 family of proteins consisting of $p15^{INK4a}$, $p16^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, and the Cip/Kip family, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (Sherr and Roberts, 1999). The INK4 proteins specifically inhibit the cyclin D-dependent kinases, Cdk4 and Cdk6, while the Cip/Kip family have a broader specificity, regulating cyclin D-, cyclin E- and cyclin A-dependent kinase activity. CKIs are regulated in a cell-type specific manner depending on the cells requirements for proliferation or quiescence, in mammalian myoblasts up-regulation of p21 mediates cell cycle withdrawal on terminal differentiation.

While the Cip/Kip proteins inhibit the cyclin E-Cdk2 complex, they appear to positively regulate cyclin D-Cdk4/6, showing only weak inhibition of kinase activity. Efficient association of cyclin D with Cdk4/6 requires the presence of Cip/Kip, and the active Cdk complex appears to be associated with a Cip/Kip protein (Ekholm and Reed, 2000; Sherr and Roberts, 1999). It is thought that cyclin D-cdk4/6 switches between an active Cip/Kip bound form and an inactive INK4 bound form, depending on the relative concentrations in the cell of the two classes of CKIs, competing for binding to the Cdk complex. In addition to facilitating assembly of the active Cdk complex, the binding of Cip/Kip proteins to cyclin D-cdk4/6 serves a second purpose. The binding and

sequestration of p27 by cyclin D-cdk4/6 releases cyclin E-cdk2 from the inhibitory effects of the CKI, which enables active cyclin E-cdk2 to carry out necessary functions for cell cycle progression, and also positively regulate its own activity by phosphorylating p27, thus marking it for degradation (Sherr and Roberts, 1999). Increased levels of INK4 proteins, such as p16, block cyclin D-dependent kinase activity by associating with Cdk4 and Cdk6, preventing their binding to cyclin D and displacing Cip/Kip proteins (Serrano, 1997). The INK4 mediated G1 arrest thus also depends on the presence of Cip/Kip proteins, which are made available to bind and inhibit cyclin E-Cdk2. Cell cycle arrest induced by INK4 proteins is abrogated in the absence of Rb, supporting the observation that a primary function of Cdk4/6 complexes is the phosphorylation and inactivation of Rb (Lukas et al., 1995; Medema et al., 1995). In Rb-/- cells, the loss of Rb is accompanied by an increase in cyclin E-cdk2 which may be sufficient to overcome inhibition by Cip/Kip proteins, driving the cell cycle forward despite the inactivation of Cdk4 and Cdk6.

1.2.2.3 Regulation of cell cycle withdrawal during myogenic differentiation

Skeletal muscle differentiation proceeds by a temporally regulated series of events comprising withdrawal from the cell cycle, expression of muscle-specific proteins and fusion to form post-mitotic multinucleate myotubes (Walsh and Perlman, 1997). Withdrawal from the cell cycle during myogenic differentiation requires coordinate regulation between MRFs and cell cycle regulators (Kitzmann and Fernandez, 2001; Lassar et al., 1994). Both positive and negative regulators of the cell cycle play a role, and many of the proteins described above as mediating the G1/S transition in dividing cells also become key players in events during the initiation and maintenance of irreversible withdrawal from the cell cycle as a critical step in myogenic differentiation. Positive regulators of the cell cycle such as E2F and cyclin-Cdk complexes inhibit myogenic differentiation while the negative regulators, Rb and CKIs, are essential for establishing and maintaining the post-mitotic state. These factors are involved in the balance between proliferation and differentiation (Olson, 1992). The vast array of literature on this field testifies to the complexity of the molecular mechanisms governing myogenic differentiation and cell cycle regulation, and the overview presented here aims to extract the salient features of these mechanisms, rather than covering the subject exhaustively. Figure 1-3 shows a general schematic of the crosstalk between cell cycle

and myogenic regulatory proteins, highlighting the most significant points of interaction, which are described in more detail below.

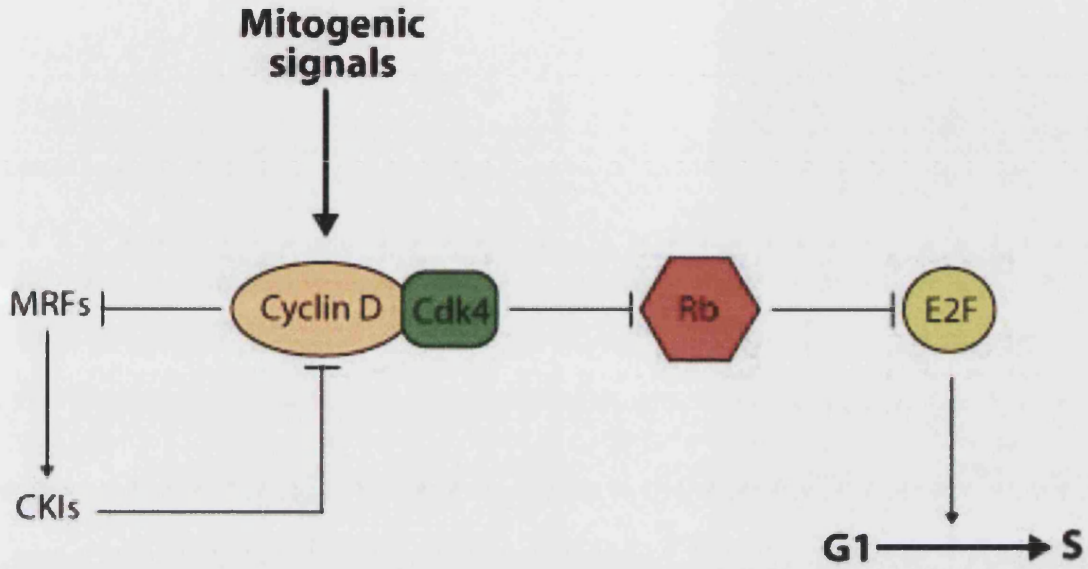


Figure 1-3: Interplay between cell cycle and myogenic regulatory factors. In the presence of mitogenic stimulus, levels of cyclin D are elevated and the cyclin D-Cdk4 complexes inactivates Rb through phosphorylation, leading to the release of E2F family transcription factors which are then free to transcribe the genes required for the cell to pass through G1 of the cell cycle and into S phase. High levels of cyclin D-Cdk4 also inhibit the MRFs (myogenic regulatory factors) such as MyoD, which is expressed in proliferating myoblasts. When mitogens are withdrawn, cyclin D is degraded and the MRFs are able to activate the transcription of genes required for myogenic differentiation, including CKIs (cyclin-dependent kinase inhibitors) such as p21, which mediate withdrawal from the cell cycle by further inhibiting Cdks, generating a feedback loop which ensures that Rb remains active and the cells remain permanently withdrawn from the cell cycle.

Active Rb has been shown to be essential for the maintenance of cell cycle arrest in myotubes (Gu et al., 1993; Schneider et al., 1994), and also for the expression of late markers of muscle differentiation through activation of MEF2 (Novitch et al., 1996; Novitch et al., 1999). Cyclin D1 and MyoD provide opposing signals for the control of Rb and relative levels of the two regulators can be thought of as determining the balance between myogenic differentiation and proliferation. One, or often both, of the primary MRFs, Myf-5 and MyoD are expressed in proliferating myoblasts in culture. As discussed above, the MRF family of transcription factors promote myogenesis and heterodimerise with E proteins to form an active complex which then binds to E box sites in the promoter regions of muscle-specific target genes (Puri 2000). As differentiation only occurs after cells have withdrawn from the cell cycle in response to decreased

availability of mitogens, the activity of Myf-5 and MyoD must be repressed in the proliferating cells.

Cyclin D1 is known to be the major G1 cyclin responsible for promoting progression through the cell cycle into S phase, and is the mitogen sensor for the cell. Under growth-promoting conditions, cycling myoblasts have high levels of nuclear cyclin D1 and its binding partner Cdk4 (Zhang et al., 1999a). Upon mitogen withdrawal, cyclin D1 is rapidly degraded, levels decrease as the cells undergo differentiation (Shen et al., 2003; Skapek et al., 1995), and in myotubes cyclin D1 is absent while Cdk4 becomes localised to the cytoplasm (Wei and Paterson, 2001). Over-expression of cyclin D1 was shown to inhibit myogenesis and correlated with increased phosphorylation of MyoD, this inhibition could be overcome by subsequent over-expression of the CKIs p16 or p21 suggesting that cyclin D-dependent kinase activity was responsible for hyperphosphorylation of MyoD (Guo and Walsh, 1997; Skapek et al., 1995). The inhibition of myogenesis by Cdks was accompanied by the release of E2F implicating the Rb pathway in the regulation of myogenesis (Guo and Walsh, 1997), however, the inhibition of MyoD activity was also shown to occur independently of Rb (Skapek et al., 1996) suggesting that a direct interaction between cyclin D-cdk4 and MyoD may also occur. While a possible role for Cdks in mediating the turnover of MyoD by direct phosphorylation has been proposed (Kitzmann and Fernandez, 2001; Puri and Sartorelli, 2000; Wei and Paterson, 2001), kinase-defective Cdk4 retains the ability to interact with MyoD, suggesting an alternative purpose for the interaction in controlling differentiation (Zhang et al., 1999a).

The interaction between MyoD and Cdk4 inhibits the DNA binding and transcription activation ability of MyoD, and is controlled by the levels of cyclin D1, which is required to shuttle Cdk4 into the nucleus (Zhang et al., 1999a). Upon differentiation, cyclin D1 is degraded and Cdk4 remains in the cytoplasm. The binding of Cdk4 to MyoD also inhibits the activity of Cdk4 (Zhang et al., 1999b), and so the relative levels of cyclin D1 and MyoD determine the balance between proliferation and differentiation (Kitzmann and Fernandez, 2001; Wei and Paterson, 2001).

The onset of differentiation is marked by the expression of myogenin and the CKI p21, in myoblasts, prior to fusion (Andres and Walsh, 1996). The induction of Cdk inhibitors is an important step in establishing cell cycle withdrawal and once induced a number of feedback mechanisms within the cell ensure that the cells remain arrested (Lassar et al., 1994; Walsh and Perlman, 1997). The MRFs promote p21 expression, which in turn inhibits Cdk activity (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). As described earlier, cyclin-dependent kinases antagonise myogenic differentiation and drive cells through the cell cycle by hyperphosphorylating Rb (Figure 1-3). Maintained in its active hypophosphorylated form, by the action of CKIs, Rb is able to sequester the E2F family of transcription factors, preventing cell cycle progression, and also cooperates with MyoD in promoting the activation of MEF2 which is required for transcription of late muscle markers (Novitch et al., 1999). Thus the myotubes maintain high expression levels of p21 even when the cells are exposed to mitogens, leading to the establishment of a terminal cell cycle withdrawal (Andres and Walsh, 1996; Walsh and Perlman, 1997).

1.2.3 Myoblast fusion

Compared with other aspects of myogenesis such as proliferation, commitment and cell cycle arrest, relatively little is known about the specific molecules which mediate the process of cell fusion. The formation of a multinucleate syncytium from committed post-mitotic mononucleates is one of the defining features of myogenic differentiation and is vital to the function of mature myofibres. In addition to the formation of muscle during development, there is continued fusion of myoblasts, generated by the activation and division of satellite cells, with existing myofibres throughout adult life in response to changes in muscle size brought about by growth or exercise, or during regeneration of damaged muscle (Schultz, 1989).

Fusion is a distinct part of the temporally regulated series of events comprising myogenic differentiation (Andres and Walsh, 1996), and is itself a multi-step process involving migration and specific recognition of other fusion competent myoblasts, followed by adhesion and alignment, and ultimately membrane fusion to form a multinucleate cell (Knudsen, 1991; Nameroff and Munar, 1976). Studies examining the migration and alignment of myoblasts have observed that fusion happens almost exclusively in an end-to-end manner rather than between adjacent membranes of myoblasts lying side by side

(Clark et al., 1997; Fear, 1977; Lipton and Konigsberg, 1972), suggesting that it occurs between specific regions of the membrane.

That the events of fusion represent distinct and separable steps, is emphasised by the discovery of conditions and molecules which can block specific aspects of the process (Knudsen, 1991). For example, EGTA prevents the specific adhesion of myoblasts but does not block the formation of fusion-competent cells, while cytochalasin B or colchicine block membrane fusion without inhibiting adhesion. The progression from adhesion to membrane fusion is marked by the acquisition of resistance to dissociation with trypsin (Knudsen and Horwitz, 1977).

Intuitively, membrane proteins are likely candidates for a role in a process which intimately involves cell-cell recognition, adhesion and membrane fusion. Fusion only occurs between myoblasts, they do not fuse with other cell types, underlining the specific nature of the interactions; when studied in suspension, myoblasts aggregate together and exclude fibroblasts (Knudsen and Horwitz, 1977). Early experiments comparing labelled proteins isolated from membrane preparations of proliferating and fusing myoblasts by gel electrophoresis, revealed differences in the proteins present at these distinct stages of differentiation (Wakelam, 1985) although the identity of many of the proteins remained unknown. Tunicamycin (Gilfix and Sanwal, 1980) and phospholipase C (Nameroff and Munar, 1976) both prevented fusion of myoblasts establishing the requirement for glycoproteins and GPI-anchored proteins respectively, on the cell surface. Since then, a number of cell surface molecules have been shown to be essential for myoblast fusion.

ADAM12 (a disintegrin and metalloprotease), also known as meltrin-α is a cell surface protein expressed in skeletal muscle and implicated in fusion. It was found to be up-regulated during differentiation of the C2C12 skeletal muscle cell line and knocking out the protein with an ADAM12 antisense molecule abolished fusion (Yagami-Hiromasa et al., 1995). How the protein regulates fusion is unknown although it has a role in processing growth factors such as HB-EGF (Cao et al., 2003). A number of different integrins may also regulate the fusion process; VLA-4, its counter receptor VCAM1 and β-1 integrin have all been specifically implicated (Rosen et al., 1992; Schwander et al., 2003).

Other specific proteins with a suspected role in fusion include calpain, a calcium-dependent protease. Calpain has been detected on the surface of myoblasts and at focal adhesions and may degrade integral membrane proteins and cytoskeletal elements, in order to destabilise the plasma membrane so that fusion can occur (Dedieu et al., 2004; Dourdin et al., 1999; Horsley and Pavlath, 2004). A number of other calcium-dependent proteins, such as M-cadherin, are likely to be involved in fusion, explaining, at least in part, the requirement of extracellular calcium for adhesion and fusion of myoblasts (Horsley and Pavlath, 2004; Knudsen, 1991; Wakelam, 1985). An increase in intracellular calcium is also needed for fusion, suggesting that the process may be regulated by calcium-dependent signalling pathways. Molecules such as prostaglandins, which lead to increases in intracellular calcium, and ion channels which mediate the influx of calcium, have been shown to play a role in fusion.

Prostaglandins are secreted proteins, as is IL-4, a molecule also implicated in fusion. The significance of secreted molecules in fusion was first realised when it was discovered that conditioned medium taken from around myoblasts was capable of enhancing fusion at cell densities below that normally required for efficient fusion (Konigsberg, 1971). Such experiments demonstrated that high cell density promoted fusion by means other than just increased cell contact, the combined depletion of growth factors, and secretion of proteins, by proliferating myoblasts produced conditioned medium which favoured differentiation and fusion (Doering and Fischman, 1977).

Rearrangements in the cytoskeleton also appear to be involved in myoblast fusion and the morphological changes which accompany it. Prior to fusion, mononucleate myoblasts elongate and align through a process driven by the reorganisation of microtubules (Bulinski and Gundersen, 1991). The radial microtubule array, nucleated from a discrete microtubule-organising centre, the centrosome, in proliferating myoblasts, is replaced by a linear alignment of microtubules running parallel to the emerging long axis of the cell (Musa et al., 2003; Saitoh et al., 1988; Warren, 1974). Treatment of myoblasts with agents which interfere with the dynamics of microtubules have been reported to block fusion. Colchicine, a microtubule-depolymerising molecules, has been shown to prevent fusion (Bischoff and Holtzer, 1968) and to prevent elongation of pre-fusion myoblasts (Saitoh et al., 1988), additionally, the microtubule-

stabilising agent taxol has also been reported to prevent fusion of post-mitotic myoblasts in culture (Antin et al., 1981).

Although the exact role of microtubules in the fusion process is not known, some of the proteins specifically associated with fusion may play a role in the cytoskeletal reorganisation providing a link between the elongation of myoblasts and the specific events of fusion. As mentioned above, M-cadherin has been implicated in fusion (Kaufmann et al., 1999; Zeschnigk et al., 1995). In proliferating myoblasts, the cell surface distribution of M-cadherin is much more seriously disrupted by actin depolymerisation than by microtubule depolymerisation, while the opposite is true in differentiating myoblasts suggesting that the adhesion molecule becomes associated with microtubules specifically during differentiation. M-cadherin may be involved in the recognition between myoblasts prior to fusion and it has been speculated that such recognition events, induced by differentiation, between microtubule-associated M-cadherin on the cell surface may mediate the alignment of microtubules within the cell (Kaufmann et al., 1999).

Myogenic differentiation involves the coordinated regulation firstly of muscle-specific transcription factors for specification of the muscle lineage, then of cell cycle regulators to mediate a post-mitotic arrest and finally molecules which promote cell recognition and membrane fusion. This carefully regulated process can be recapitulated in culture using myogenic cell lines which show the same temporal regulation of events. Many cell types undergo withdrawal from the cell cycle on differentiation, but skeletal muscle additionally undergoes a mononucleate to multinucleate transition resulting in the formation of post-mitotic multinucleate myotubes. During urodele limb regeneration, the cells at the site of the amputation, including mature myofibres, are able to dedifferentiate and contribute to the proliferating mass of the blastema. For skeletal muscle fibres, this involves not only re-entry into the cell cycle but also a return to the mononucleate state. During differentiation, cell cycle withdrawal and the mononucleate to multinucleate transition are distinct separable events and these two aspects of the dedifferentiation process will now be described separately, although links between the two are discussed. In keeping with the earlier considerations made to the plasticity of the differentiated state

in mammalian systems, experimental evidence for dedifferentiation in both urodeles and mammals will be covered.

1.3 CELL CYCLE RE-ENTRY

1.3.1 S phase re-entry in urodele myotubes

Post-mitotic arrest in mononucleate myoblasts is an early event in myogenic differentiation and marks a permanent withdrawal from the cell cycle for mature mammalian muscle cells, both *in vivo* and in culture. The presence of growth factors such as FGF and TGF-β are known to inhibit myogenesis and promote the continued proliferation of myoblasts (Clegg et al., 1987; Vaidya et al., 1989), following fusion however, the multinucleate myotubes are refractory to growth factors. This is thought, in part, to be due to the loss of receptors on the cell surface as part of the differentiation process, rendering the cell unresponsive to mitogens in the surrounding environment (Hu and Olson, 1990; Olwin and Hauschka, 1988). In both murine and newt muscle cell lines in culture, myotubes fail to re-enter the cell cycle in response to a wide range of growth factors usually present in serum, and which successfully stimulate proliferation of the mononucleate myoblast population in the same culture (Tanaka et al., 1997).

However, experiments carried out *in vitro* revealed that newt myotubes, while refractory to a range of individual growth factors, re-entered the cell cycle in response to stimulation with high serum from all sources (Tanaka et al., 1997). Although the identity of the factor responsible for bringing about the response remains to be elucidated, this work revealed that the unknown factor, present in serum, required prior exposure to thrombin. Treatment with thrombin enhanced the ability of serum to stimulate S phase re-entry in the newt myotubes to the extent that nuclei were activated at sub-threshold serum concentration, preparations of crude thrombin were also found to contain the activity (Tanaka et al., 1999). When crude thrombin was fractionated by protein chromatography, the active fraction was found to act specifically on myotubes, inducing S phase re-entry while having no effect on mononucleate myoblasts. Mammalian myotubes remained unresponsive to this activity. Thrombin is a serine protease, produced by cleavage of its inactive precursor pro-thrombin, and is one of the key enzymes activated as part of the coagulation cascade in response to wounding (Coughlin,

1999). Thrombin activity was found to be up-regulated in the limb blastema and has also been found to be up-regulated at the dorsal margin of the newt iris following lentectomy (Imokawa and Brockes, 2003; Imokawa et al., 2004), implicating the protease in regeneration of both the limb and lens and suggesting a possible common mechanism for regeneration in response to injury.

As discussed previously, Rb is a key regulator of the cell cycle arrest which occurs during myogenesis, and the phosphorylation state of the protein controls progression through the cell cycle. In myotubes, Rb is maintained in an active hypophosphorylated state but when newt myotubes were induced to undergo S phase re-entry by stimulation with serum, Rb was found to be phosphorylated (Tanaka et al., 1997) suggesting that fundamental differences in the way in which Rb is regulated may account for the disparate behaviour of newt and mammalian myotubes in response to serum. Additionally, a cell density dependent aspect to S phase re-entry was described adding a further level of complexity to the mechanism involved in maintaining cell cycle arrest in differentiated muscle. When newt myotubes were cultured at high density they were unresponsive to the thrombin-mediated activity (Tanaka et al., 1997) suggesting that S phase re-entry can also be modulated via signals generated through cell-cell contact. It should be noted that after entering S phase, nuclei failed to continue progressing through the cell cycle and arrested before mitosis.

In addition to investigations carried out on newt myotubes in culture, a number of experiments have described the implantation of cultured newt myotubes into the blastema following amputation of a urodele limb (Kumar et al., 2000; Lo et al., 1993; Velloso et al., 2000). Newt myoblasts were fused in culture and the myotubes labelled by either incorporation of tritiated thymidine into myoblast nuclei prior to fusion, injection of myotubes with a fluorescent lineage tracer dye, or prior infection with a pseudotyped retrovirus. Labelled myotubes were implanted and the blastemas harvested at various timepoints over the following weeks. These experiments provided an excellent means to follow the behaviour of myotubes in a regeneration permissive environment and added convincing evidence to the case for dedifferentiation during regeneration. As early as one week after implantation into the blastema, labelled mononucleates were seen in the blastema and the number of these labelled cells increased

over time suggesting that the cells were actively proliferating (Lo et al., 1993). Incorporation of BrdU or tritiated thymidine into these cells confirmed that DNA synthesis was occurring in these cells (Velloso et al., 2000). A series of experiments using newt myotubes labelled with a genetic marker provided a particularly clear example of these events. The cells, identifiable by stable expression of human alkaline phosphatase, transfected into the cell by infection with a pseudotyped retrovirus, were implanted into blastemas. Nine days post-implantation, the animal was injected with BrdU and the blastema analysed for double-labelled cells 24 hours later (Kumar et al., 2000). The genetic marker provided a more reliable and persistent means of following cells and revealed that 42-86% of alkaline phosphatase-expressing myotubes contained two or more BrdU positive nuclei. In addition, some BrdU incorporation was seen in the nuclei of host myofibres at the level of amputation, suggesting that the implanted myotubes are indeed illustrating physiologically relevant events.

Importantly, the implantation experiments demonstrated that the phenomenon of S phase re-entry observed in cultured newt myotubes when exposed to high levels of serum, also took place when the myotubes were exposed to signals in the regenerating blastema. The cellularisation of multinucleate myotubes giving rise to mononucleate progeny is an aspect of dedifferentiation which will be discussed in more detail later in this chapter.

1.3.2 S phase re-entry in mammalian myotubes

While newt myotubes in culture readily respond to the presence of high levels of serum by re-entering S phase, mammalian myotubes maintain their post-mitotic arrest in response to both specific growth factors and serum, and to the thrombin-mediated activity detailed above for its effect on newt myotubes (Tanaka et al., 1997). However reversal of the cell cycle arrest in mammalian muscle cultures has been described following gene knockout or infection with DNA tumour virus proteins. These experiments once again implicate the retinoblastoma protein as a key regulator of the differentiated state.

Several groups generated Rb knockout mice and all reported similar findings (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Rb+/- heterozygotes developed apparently completely normally but all Rb-/- mice died *in utero* and displayed a range of diverse

abnormalities. Surprisingly, the muscle of these embryos appeared grossly normal suggesting that Rb is not essential for muscle development at least up until embryonic day 14-15, the latest stage achieved by the null mice. Instead, the defects were predominantly in neural and haematopoietic development with severe disruption of erythropoiesis, and aberrant mitoses and cell death in the neuronal system.

In many cell types, as in muscle, proliferation and differentiation are mutually exclusive activities for the cell (Olson, 1992), so Rb is likely to play a role in the terminal differentiation of many cell types (Coppola et al., 1990). Other pocket proteins can reportedly compensate for some of the functions of Rb (Mulligan and Jacks, 1998; Schneider et al., 1994) but the severity of the phenotype in the Rb knockout mouse clearly demonstrates that Rb is essential for survival.

As the Rb knockout mouse is embryonic lethal, Rb-/- cell lines have been valuable tools to investigate the role of the protein in terminal differentiation. Myogenic cells lacking the Rb gene were found to form normal myotubes but, strikingly, the nuclei were seen to re-enter the cell cycle when exposed to high levels of serum (Schneider et al., 1994). Examination of other pocket proteins revealed that p107, which is normally expressed only in myoblasts, was up-regulated in the Rb-/- myotubes, supporting the idea of some redundancy among this family of proteins. However, upon challenge with serum, p107 was deinduced, levels of the protein decreased and the cell nuclei re-entered S phase. A similar failure to maintain the post-mitotic block was seen in other Rb-/- lineages; adipocytes undergo terminal withdrawal from the cell cycle during differentiation and in the absence of the Rb gene were also observed to re-enter S phase when exposed to serum (Schneider et al., 1994). This showed that while p107 can compensate for Rb in establishing a cell cycle block at the onset of myogenesis, Rb alone is responsible for maintaining this block in mammalian myotubes in the presence of serum.

Rb also interacts with MyoD to mediate myogenesis and it has been reported that in the absence of Rb, MyoD-regulated gene expression is diminished causing poor induction of late markers of muscle differentiation such as MHC, although early markers are unaffected (Novitch et al., 1996). The nuclei in serum-stimulated Rb null myotubes underwent DNA synthesis and accumulated in S and G2 but did not enter mitosis or

undergo cytokinesis (Novitch et al., 1996; Schneider et al., 1994), in parallel with the behaviour of newt myotubes in culture, suggesting that S phase re-entry as a result of the loss or inactivation of Rb is not sufficient to induce further progression through the cell cycle in terminally differentiated muscle.

In addition to Rb null myotubes, S phase re-entry has also been observed in myotubes infected with a number of DNA tumour viruses. SV40 large T antigen is known to have oncogenic potential and can transform cells. Introduction of the large T antigen into primary myoblasts via a retroviral vector immortalised the cells but allowed them to fuse and express muscle-specific markers when induced to differentiate (Iujvidin et al., 1990). The myotube nuclei did not withdraw from the cell cycle and mitotic figures could be seen. Use of an inducible SV40 large T antigen has allowed terminal differentiation of a mouse myogenic cell line in the absence of the oncogene, followed by induction of the gene in the mature myotube (Endo and Nadal-Ginard, 1989). Cell cycle re-entry was observed by measuring incorporation of tritiated thymidine and within 24hrs over 90% of the myotube nuclei had entered S phase, this was followed by the appearance of mitotic nuclei. While there have been mixed reports on the eventual outcome of these mitotic myotubes, all agree that in some of the myotubes the observed cell cycle re-entry is lethal, nuclei become enlarged and deformed, eventually resulting in degeneration and apoptosis of the myotube (Endo and Nadal-Ginard, 1989; Endo and Nadal-Ginard, 1998; Iujvidin et al., 1990). However, in some myotubes organisation of condensed chromosomes within a mitotic spindle were observed and in a minority of cases, cells containing a single nucleus budded off from the main body of the myotube (Endo and Nadal-Ginard, 1989). Myotubes were also seen to fragment giving rise to two smaller myotubes by a process reminiscent of cytokinesis, with a midbody visible as intense β-tubulin staining, and a contractile actin ring at the site of cleavage (Endo and Nadal-Ginard, 1998).

Similar results were reported in mammalian myotubes expressing the adenovirus E1A oncogene. Adenoviruses are of particular utility for gene delivery in studies of differentiated cell types as they are capable of infecting post-mitotic cells. Infection of mammalian myotubes and expression of the E1A adenoviral protein in myotubes resulted in the incorporation of BrdU into myotube nuclei (Crescenzi et al., 1995; Latella et al.,

2000). As in the presence of SV40 large T antigen, many of the nuclei progressed through the cell cycle undergoing mitosis, and in some cases fragmentation of the myotube occurred. Ultimately, the myotubes underwent apoptosis at the next mitosis, but terminally differentiated mononucleate cells which had failed to fuse were reportedly able to undergo multiple rounds of mitosis both in the presence and absence of alternate cycles of cytokinesis (Crescenzi et al., 1995; Latella et al., 2000).

Direct interaction between the Rb protein and viral oncogenes, including adenovirus E1A and SV40 large T antigen was demonstrated even before the role of Rb in cell cycle regulation or myogenesis was fully understood (DeCaprio et al., 1988; Whyte et al., 1988). In fact, the discovery that DNA tumour viruses bound to Rb provided valuable information on the mechanism of action of Rb and contributed to the elucidation of its role in cell cycle progression (Lukas et al., 1994; Nevins, 1992). Viral proteins commonly target the mechanisms which regulate cell cycle progression, in order to activate and exploit the host cells proliferative machinery for its own replication. In its active hypophosphorylated conformation, Rb binds to and sequesters E2F, a family of transcription factors required for the transcription of genes involved in growth and DNA replication. Viral oncoproteins disrupt the association of Rb and E2F, effectively competing with E2F for binding to the Rb pocket domain and releasing the transcription factor to advance the cell cycle (Nevins, 1992). Binding of viral oncoproteins to Rb also completely abrogates the requirement of cyclin D1 for progression through G1; the cyclin D1-Cdk4/6 complex normally functions to phosphorylate and inactivate Rb in mid-late G1 phase (Lukas et al., 1994). The E1A and SV40 large T antigen oncoproteins also disrupt the binding of other proteins which interact with Rb.

Inactivation of the Rb protein by these viral oncogenes is in agreement with the results described for Rb null myotubes and newt myotubes in culture, where Rb is presumably inactivated by endogenous Cdks, and confirms the vital role of Rb in mediating terminal withdrawal from the cell cycle during myogenesis. The more extensive effects of the oncogenes, including progression through S phase and G2 into mitosis, fragmentation of the myotubes and high levels of apoptosis, can be attributed to the fact that these viral proteins do not bind exclusively to Rb but can sequester the whole pocket protein family and other cell cycle regulated proteins including cyclin A, cyclin E and cdk2 (Nevins,

1992). The exact cause of cell death is unclear and could occur via a cellular mechanism designed to induce apoptosis upon inappropriate entry in M phase.

While these studies with viral oncoproteins demonstrate the potential for S phase re-entry in mammalian myotubes, the complex interactions with numerous intracellular proteins means they provide only limited information about the details of the molecular mechanisms involved. To address these issues, over-expression of key cell cycle regulators in post-mitotic myotubes has been used to try and stimulate S phase re-entry in mammalian myotubes and so isolate which targets of the viral oncoproteins are responsible for this activity. In this way, cyclin D-dependent kinase activity has been shown to play a vital role in regulation of the cell cycle in multinucleate myotubes.

Over-expression of E2F can induce quiescent myoblasts to re-enter the cell cycle but was insufficient to stimulate myotubes, suggesting that the post-mitotic block in these cells represents a more stable and tightly controlled arrest (Pajalunga et al., 1999). In these myotubes Rb failed to be phosphorylated but although E2F was not sufficient to induce S phase re-entry, it was found to be necessary for the action of the adenovirus E1A protein demonstrating that the activation of the E2F family of transcription factors is an important part of the mechanism by which viral oncoproteins drive myotubes into S phase (Pajalunga et al., 1999). Cyclin E and cdk2 also failed to initiate S phase, apparently due to accompanying increases in the levels of cdk inhibitors (Latella et al., 2001).

In contrast, over-expression of cyclin D1 and cdk4, was able to stimulate S phase re-entry in mammalian myotubes suggesting that cyclin D-associated kinase activity is a major target of the cellular machinery responsible for maintaining the cell cycle arrest in myotubes (Latella et al., 2001). The Cyclin D/cdk4 complex is the major kinase responsible for phosphorylating Rb and plays a significant role in regulating cell cycle progression at the G1/S checkpoint. In support of these findings, earlier work showed that while knockout of cyclin D1 prevented entry into S phase in cycling cells, viral oncoproteins, or mutations in the Rb gene bypassed the cells requirement for cyclin D-associated kinase activity, driving cells into S phase even in the absence of cyclin D1 (Lukas et al., 1994).

In addition to DNA tumour viruses a further example of S phase re-entry in terminally differentiated myotubes, which have not been previously manipulated, exists in the nematode *Trichinella spiralis*, though the mechanism is poorly understood compared to that mediated by viral oncogenes. This intracellular parasite selectively infects mammalian skeletal muscle and has been shown to bring about dramatic changes in the morphology of myofibres including the appearance of a characteristic collagen capsule, enlarged nuclei and loss of expression of myofibrillar proteins. In experimentally infected mice, incorporation of tritiated thymidine revealed that S phase re-entry within the myofibre is an early event, with ~85% of nuclei labelled between 2 and 5 days post-infection and within 20 days, levels of muscle-specific transcription factors, MyoD and myogenin, and proteins such as MHC and creatine kinase were massively reduced (Jasmer, 1993). These nuclei then remained stably blocked in S/G2 with an approximately 4N DNA content for at least 60 days after infection. The mechanism by which *T. spiralis* induces cell cycle re-entry is currently unclear and the role, if any, of Rb in these events is unknown

Finally in this discussion of the reversal of cell cycle arrest in mammalian myotubes, are the findings from creation of newt-mouse hybrid myotubes. Earlier heterokaryon experiments demonstrated the plasticity inherent in terminally differentiated mammalian cells, and revealed that the tissue-specific gene expression patterns of a given cell type are not fixed and can be altered by cytoplasmic elements. As a cell becomes committed to a particular lineage, the potentiality of that cell narrows and the cell becomes progressively unresponsive to the signals specifying alternative cell fates. This is also true of cell lines in culture which can often require genetic intervention to manipulate the cells down a different pathway. When heterokaryons were formed by the fusion of myoblasts with other cell types such as keratinocytes, chondrocytes and hepatocytes, experimenters discovered the expression of muscle-specific genes in non-muscle nuclei (Blau et al., 1985; Clegg and Hauschka, 1987). These experiments challenged the view that once committed, nuclei could not be reprogrammed, and clearly demonstrated that during differentiation, the genes required for alternative lineages are not irreversibly inactivated and that nuclei can respond when exposed to elements in the cytoplasm of another differentiated cell type, after fusion (Blau et al., 1985).

Myoblasts from mouse and newt myogenic cell lines were fused in culture and a proportion of the myotubes formed contained nuclei from both species (Velloso et al., 2001). Given the discovery, discussed above, that newt myotubes undergo S phase re-entry in response to serum stimulation, the hybrid myotubes were similarly stimulated to determine whether mouse nuclei, in this unique cytoplasmic environment, could also respond. Results showed that in almost 10% of the hybrid myotubes, mouse nuclei had re-entered the cell cycle (Figure 1-4). This work demonstrated that there is considerable potential for plasticity of the differentiated state even in mammalian cells and confirmed that the mouse nuclei inherently retained the ability to respond to activation of the newt pathway.

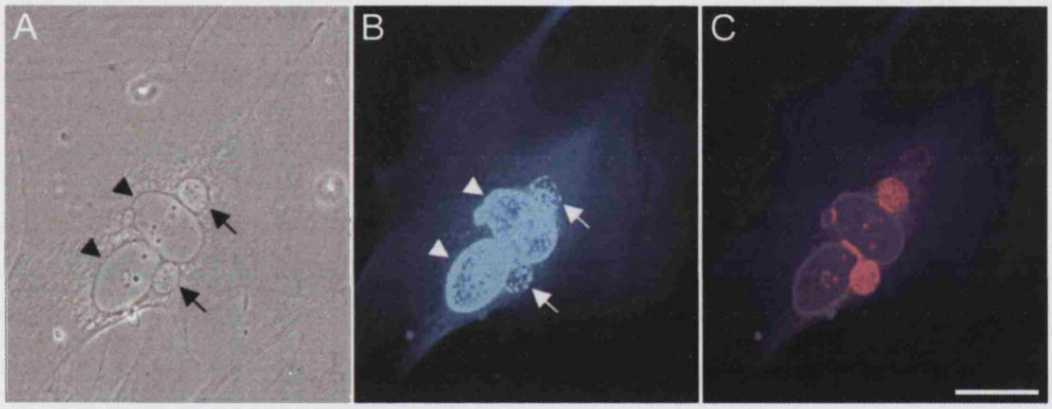


Figure 1-4: Mouse nuclei respond to serum and re-enter S phase in a newt/mouse hybrid myotube. Phase contrast (A) and fluorescent images (B, C) of a newt/mouse hybrid myotube; MHC staining is shown in blue and BrdU staining in turquoise, mouse laminin staining in red identifies the murine nuclei. Scale bar = 50μm. Newt and mouse myoblast cell lines were fused in culture to generate hybrid myotubes containing nuclei of both species; mouse nuclei are indicated with arrows and newt nuclei with arrowheads. When the cells were stimulated with high serum, both mouse and newt nuclei were observed to re-enter the cell cycle, as determined by incorporation of BrdU. In culture, newt myotubes are able to re-enter S phase in response to high serum, while mammalian myotubes remain completely refractory. This work established that mammalian post-mitotic nuclei retain the ability to respond to serum and can be re-programmed when exposed to signals transmitted through the newt cytoplasm. (From Velloso et al; 2001).

1.3.3 Conclusions from S phase re-entry in myotubes

The experiments described in this section have specifically addressed the issue of post-mitotic arrest during myogenesis and the extent to which this aspect of terminal differentiation is reversible in both urodele and mammalian cells. The differences which are clearly apparent between the two systems highlights that understanding the regulation of this aspect of differentiation may provide valuable insights into the mechanisms which allow the newt to regenerate while the mouse cannot. However, there are also some

parallels to be drawn which can shed light on which elements are likely to be key players in the events of regeneration.

There is clearly a difference between newt myotubes and their mammalian counterpart as demonstrated by their response to the thrombin activated factor in serum. The cell cycle arrest initiated in response to differentiation would appear to be mediated by Rb in an analogous way in the newt and mammalian system, whereby the protein becomes hypophosporylated as the cells withdraw from the cell cycle in G1, and remains in a hypophosporylated state under normal differentiation conditions. The difference between the two systems becomes apparent when the myotubes are challenged with high levels of serum. Newt myotubes react to serum in a manner reminiscent of the behaviour of Rb-/- mouse myotubes when treated with high serum but as biochemical analysis revealed, the newt myotubes do possess Rb. Analysis of newt myotubes stimulated with serum revealed that Rb, which remains permanently hypophosphorylated in mouse myotubes (Gu et al., 1993), became hyperphosphorylated in the newt cells in response to serum (Tanaka et al., 1997). That the cellular response is mimicked in mammalian myotubes which lack the Rb gene, or have been infected with viral proteins which interfere with cell cycle regulation by Rb, highlights the importance of Rb in maintaining the mitotic arrest and suggests that differences in the regulation of this protein in response to specific serum components are key to understanding the difference in the response between newt and mouse.

That newt cells should display a similar mechanism for cell cycle withdrawal during myogenesis is consistent with the need for mitotic arrest in muscle under normal physiological conditions, to enable the tissue to fulfil its function. It is only in response to injury that a regenerative response need be activated so it is important that there are specific signals which ensure that dedifferentiation only occurs at the appropriate time. High serum levels and activated thrombin would only be encountered by muscle cells following wounding, so in culture, treatment of newt myotubes with serum may mimic an injury response. The exact identity of the thrombin-mediated activity remains to be elucidated but the observation that the activity responsible for cell cycle re-entry in newt myotubes is present in mammalian serum, and may be generated in response to injury

provides optimism that some, if not all, components of the mechanism of regeneration remain encoded but unused in the mammalian system.

The results with the newt myotubes are striking as they require no manipulation of the cells, representing a response of the cells to external stimuli, but the genetic manipulations and transfections of mammalian cells are nonetheless enlightening. These experiments and in particular the generation of newt-mouse heterokaryons provide compelling evidence that mammalian muscle cells retain the intracellular components required to respond to regenerative signals. However, in the case of virally infected mammalian myotubes where many of the nuclei progress through S phase and undergo mitosis, there are high levels of cell death. The accumulation of nuclei in S and G2 in newt myotubes and Rb-/- mouse myotubes suggests that inappropriate entry into mitosis is lethal for the myotubes and there may be mechanisms in place to prevent this occurring in the absence of additional signals. It should also be stressed that reversal of cell cycle arrest is only one aspect of differentiation in muscle and in the experiments described in this section, the second major index, the acquisition of a multinucleate state, was reversed only when newt myotubes were implanted into a limb blastema. This suggests there may be specific signals in the permissive environment of the blastema which are required for cellularisation and successful progression through the cell cycle. Reversal of the mononucleate to multinucleate transition and its relationship to the release of post-mitotic arrest is discussed below.

1.4 CELLULARISATION: REVERSAL OF THE MONONUCLEATE TO MULTINUCLEATE TRANSITION

A large body of evidence now testifies that regeneration of the newt limb involves dedifferentiation and that although there may be a contribution from resident tissue-specific stem cells, a large proportion of the blastemal cells derive from the dedifferentiation of tissues such as muscle and cartilage at the site of amputation. Increasingly informative experiments have been carried out over the last 50 years; from inferences made from early observations and electron microscopy studies to the application of technologies such as timelapse microscopy to follow the events of regeneration *in vivo* and in culture.

Myogenic differentiation, as described earlier, involves cell cycle withdrawal and fusion to form a multinucleate syncytium; both aspects must be reversed in order to derive proliferating, dedifferentiated, mononucleate cells capable of contributing to the regenerating limb. The reversal of the mononucleate to multinucleate transition is referred to here as cellularisation, and is used to describe the process by which mononucleate cells are formed from a multinucleate myotube. The actual mechanism of cellularisation has not been well-studied; although the process of fusion is also poorly understood it is thought to involve a number of distinct, separable steps including recognition, adhesion and membrane fusion. Some of the cell surface molecules involved in these steps are expressed only transiently during the fusion process while others are retained. It is unlikely that the cellularisation process represents a direct microscopic reversal of the fusion process but so far the genes, molecules or cellular modifications that mediate cellularisation are largely unknown.

1.4.1 Cellularisation of urodele myotubes

An early electron microscopy study (Hay, 1959) described the fine structure of cells in the limb blastema of regenerating axolotl larvae and documented the transition from multinucleate myotube to mononucleate blastema cell, accompanied by disintegration of myofibrils, formation of new plasma membrane separating the emerging mononucleate cells and acquisition of the characteristic morphology of a dedifferentiated blastemal cell. This study concluded, as had earlier work using light microscopy, that cellularisation and dedifferentiation of myofibres occurred during urodele limb regeneration.

Since then, implantation experiments have also demonstrated that the blastema provides an environment in which cultured newt myotubes can fragment and undergo reversal of the mononucleate to multinucleate transition (Kumar et al., 2000; Kumar et al., 2004; Lo et al., 1993). Newt myotubes in culture were labelled with a tracker dye or a genetic marker, followed by implantation into the blastema (Figure 1-5). The visualisation of labelled mononucleates in the blastema over the following weeks clearly showed that myotubes had undergone cellularisation and retained the capacity to reverse this aspect of differentiation when in an appropriate environment.

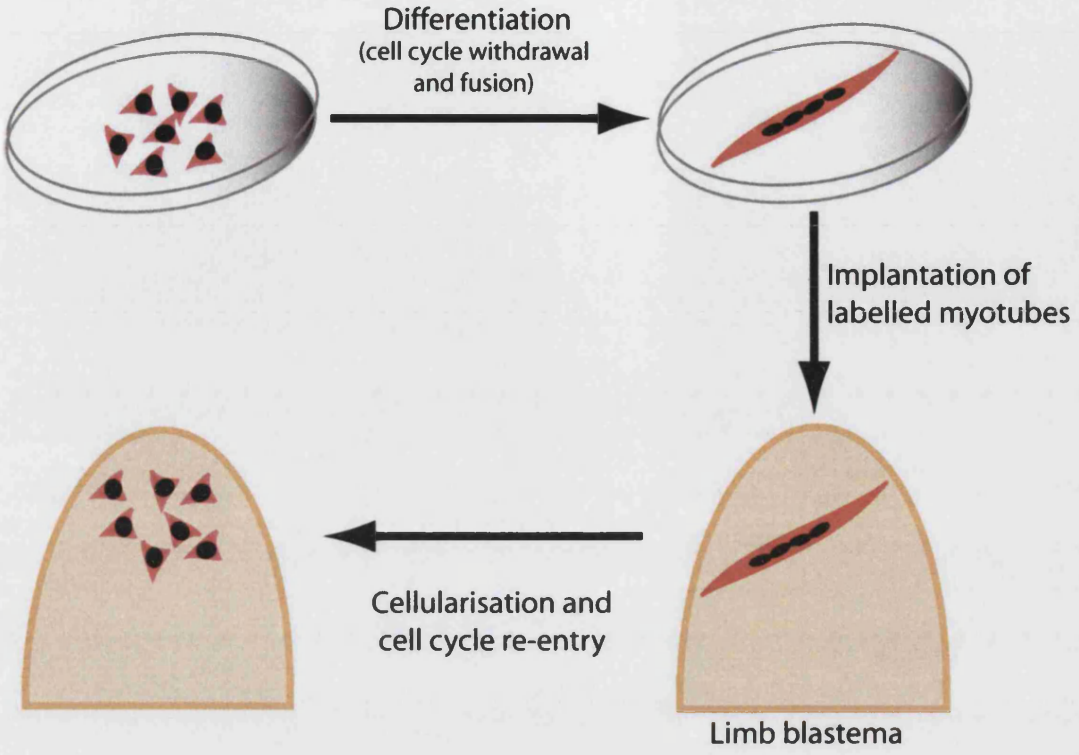


Figure 1.5: Cellularisation of newt myotubes by implantation into the blastema. Proliferating newt myoblasts were induced to differentiate in culture to form post-mitotic multinucleate myotubes. Individual myotubes were then labelled with a genetic marker or lineage tracer dye, and implanted into the blastema of an amputated newt limb. Within 7 days, labelled mononucleate cells were detected in the blastema. These experiments demonstrated the plasticity of the differentiated state during urodele regeneration and suggested that the blastema is a permissive environment for cellularisation and dedifferentiation of mature muscle.

Many of the experiments discussed in the previous section describe S phase re-entry of the nuclei in both newt and mammalian myotubes in culture but in the majority of cases the nuclei did not successfully complete mitosis and cytokinesis, and were rarely observed to undergo any process resembling cellularisation *in vitro*. Under different experimental conditions such as implantation into the limb blastema for newt myotubes (Kumar et al., 2000; Lo et al., 1993) or, occasionally, following infection with viral oncogenes for murine myotubes (Endo and Nadal-Ginard, 1998; Latella et al., 2000), the cells appear to not only have reversed the cell cycle withdrawal imposed during myogenesis but also undergo a process of cellularisation, reversing the mononucleate to multinucleate transition which occurred when myoblasts underwent fusion.

The relationship between these two aspects of the differentiated state was explored by Velloso et al (Velloso et al., 2000) using myotubes in which a permanent cell cycle block

was created using two distinct methods; x-irradiation to induce a checkpoint mediated arrest in response to DNA damage, and secondly, transfection with a plasmid expressing p16, an inhibitor of Cdk4/6 (Lukas et al., 1995; Medema et al., 1995). Expression of p16 was previously shown to block the thrombin-mediated serum response of newt myotubes by preventing the phosphorylation of Rb (Tanaka et al., 1997). Implantation of both irradiated and p16 expressing myotubes into a newt limb blastema induced the formation of mononucleate progeny in the absence of cell cycle re-entry suggesting that the mechanisms controlling these two aspects of differentiation are distinct and separable, and that S phase re-entry is not a prerequisite for cellularisation. Whether the converse is true, or whether cellularisation can ultimately induce cell cycle re-entry in the resulting mononucleates is an issue discussed to some extent later in this chapter and is also addressed in the course of experiments described in subsequent chapters of this thesis.

Initially the discovery that S phase re-entry could be induced in newt myotubes and was observed in regenerating limbs *in vivo* (Kumar et al., 2000; Lo et al., 1993), led to the belief that S phase re-entry was an early event in regeneration and that mononucleate progeny then formed by cytokinesis following mitosis. While re-entry into the cell cycle may remain an early event in dedifferentiation *in vivo* in the context of limb regeneration, this proposed mechanism for cellularisation is not consistent with the results described above and suggests an alternative pathway, perhaps involving budding of nuclei, in the absence of mitosis.

Further evidence for such a budding mechanism has come from observations of dissociated axolotl myofibres in culture (Kumar et al., 2004). In these primary cultures, a proportion of the myofibres underwent a spontaneous budding process whereby a cauliflower-like structure appeared and eventually resolved to give mononucleate cells. Using a lineage tracer dye, these mononucleates were confirmed to originate from myofibres, and measurement of the DNA content in a colony of these cells suggested they had re-entered the cell cycle and were cycling. A further proportion of the myofibres fragmented to form smaller multinucleate cells, which remained post-mitotic while the remaining myofibres appeared to be quiescent, and did not undergo cellularisation or fragmentation. The primary myofibres represent a more highly differentiated, and arguably more physiological cell type than the cultured myotubes used

in many studies, derived from a myogenic newt cell line. Implantation of labelled myofibres back into the blastema of a regenerating animal reiterated the results of previous experiments with implantation of cultured myotubes, demonstrating that these primary myofibres also undergo cellularisation and proliferation in the environment of the blastema (Kumar et al., 2004).

Limb regeneration in urodele amphibians such as the axolotl occurs by a process that involves the dedifferentiation of mature muscle fibres and these observations of axolotl myofibres in culture generated further evidence for an endogenous programme of cellularisation and dedifferentiation in these organisms, and importantly also provided an *in vitro* system in which cellularisation of myofibres from a regenerating animal could be manipulated and studied. Examination of a number of elements suspected to play a role in cellularisation and dedifferentiation in the context of regeneration revealed a role for microtubules, as taxol effectively blocked cellularisation, and for the homeobox protein Msx-1. *In situ* hybridisation revealed that Msx-1 was expressed in the nuclei of active myofibres undergoing cellularisation while the mRNA was undetectable in the population of quiescent myofibres. In addition, the use of morpholino antisense oligonucleotides to Msx-1 resulted in decreased levels of the Msx-1 protein and significant inhibition of cellularisation as measured by the percentage of active myofibres, strongly implicating Msx-1 in cellularisation and dedifferentiation of axolotl myofibres (Kumar et al., 2004).

Perhaps the most compelling demonstration of cellularisation in the urodele system has been the visualisation of axolotl myofibres *in vivo* during tail regeneration, made possible by technological advances in microscopy and the properties of the chosen axolotl larval system (Echeverri et al., 2001). Previous studies using implanted newt myotubes showed that cellularisation was possible in the blastema but still did not directly demonstrate that this was a physiologically relevant event occurring in the endogenous myofibres during regeneration.

Individual myofibres in an axolotl larva tail were injected with a fluorescent lineage tracer, following which the tail was amputated through, or near to, the labelled myofibres. The axolotl larva tail is optically clear which enabled the labelled myofibres

to be imaged *in vivo* at regular time points throughout regeneration. When amputation resulted in injury to the tip of a labelled myofibre, under conditions of massive tissue damage, labelled mononucleates could be seen between 2 and 5 days later; the number of mononucleates corresponded to the number of nuclei previously in the myofibre suggesting that the entire myofibre undergoes cellularisation in response to injury at one end. Within 48 hours after cellularisation, the number of mononucleate progeny increased suggesting that the cells were actively dividing, shortly after, they became morphologically indistinguishable from blastemal cells. Severe injury to the myofibre caused degeneration of the cell, while amputation close to, but not through, the labelled fibre was insufficient to stimulate dedifferentiation of the labelled myofibre (Echeverri et al., 2001). These experiments clearly confirmed the findings implicit in earlier work that cellularisation and dedifferentiation of multinucleate muscle fibres are physiologically relevant events, which occur during urodele regeneration and make a quantitatively significant contribution to the blastema.

1.4.2 Cellularisation of mammalian myotubes

Infection of mammalian myotubes with the viral oncoproteins SV40 large T antigen (Endo and Nadal-Ginard, 1989; Endo and Nadal-Ginard, 1998) or adenovirus E1A (Crescenzi et al., 1995), reported that myotube nuclei were not only able to re-enter S phase but could occasionally complete mitosis and undergo a process reminiscent of cytokinesis, or a budding off of nuclei, to generate mononucleate cells. However, the frequency of these events was very low, and progression through mitosis more often caused the appearance of deformed nuclei and led to apoptosis and degeneration of the myotube. In the rare cases of budding that produced mononucleate cells, the fate of these cells was unclear and no subsequent proliferation was reported. In the majority of cases, the results maintained that forced progression into mitosis by viral oncogenes under inappropriate conditions, i.e. in a multinucleate syncytium, far from inducing cellularisation, was lethal for the cell.

The model favoured by work on S phase re-entry suggested that a major difference between urodele and mammalian myotubes was a loss of responsiveness by mammalian cells to the extracellular signals required to initiate dedifferentiation and regeneration (Stocum, 1999). Reprogramming of the mammalian post-mitotic nucleus in the

cytoplasmic environment of a newt cell (Velloso et al., 2000) implied that mammalian nuclei were still capable of responding to dedifferentiation signals. In addition, cellularisation of newt myotubes did not occur in culture, only in the permissive environment of the regeneration blastema suggesting that the blastema provided cues that are absent in the mammalian injury response. Recent work investigated and challenged this model by exposing mammalian myotubes to the many proteins and signalling molecules in the blastema (McGann et al., 2001).

McGann et al prepared a regeneration extract from newt limb blastemas and used it to treat mammalian myotubes in culture. After four days, 18% of the myotubes contained BrdU positive nuclei indicating S phase re-entry, and up to 30% showed a decrease in expression of muscle-specific transcripts, in contrast to control cultures, which showed no evidence of dedifferentiation. 11% of myotubes underwent fragmentation events and in about half of these cases cellular proliferation over a subsequent 10 day period was observed. The authors concluded that this proliferation could not have been caused by the presence of contaminating mononucleates but instead arose by division of viable mononucleate progeny produced by cellularisation events, induced by the blastema extract (McGann et al., 2001). Similar effects were seen when newt myotubes in culture were treated with the regeneration extract, with 25% and 16% of myotubes undergoing S phase re-entry and fragmentation respectively. While the factor responsible for this activity has not been identified, the experiment demonstrated that cellular dedifferentiation is possible in un-manipulated mammalian myotubes by addition of an external factor(s) present in the limb blastema of a regenerating newt. These results proposed a model whereby mammalian systems have not lost responsiveness, nor is terminal myogenic differentiation an irreversible state, but instead they simply do not express the appropriate signals to stimulate dedifferentiation in response to tissue damage.

Rosania et al also reported manipulation of the differentiated state of mammalian myotubes by treatment with an external stimulus. In this case the molecule responsible was known, and rather than being a tissue-derived factor as with the blastema extract, was a chemically synthesised purine-based compound named myoseverin (Rosania et al., 2000). The authors claimed that when added to mammalian myotubes in culture,

myoseverin was capable of inducing cellularisation, giving rise to proliferating mononucleates, and with accompanying changes in gene expression suggesting a possible connection to the events of urodele regeneration. The details of these experiments and their significance in the wider context of regeneration and plasticity of the differentiated state are discussed later in this chapter.

A further report of cellularisation of mammalian myotubes, this time involving prior genetic manipulation of the cells, concerns expression of Msx-1. In this study cells from a mammalian muscle cell line were stably transfected with Msx-1 under the control of an inducible promoter, allowing conditional over-expression of the protein (Odelberg et al., 2000). Cells were first stimulated to undergo myogenic differentiation and fusion in the absence of exogenous Msx-1 expression, as expression of the transgene in mononucleate myoblasts blocked differentiation, then expression of Msx-1 in the myotubes was induced in the presence of serum. Within days, a proportion of the myotubes underwent cleavage to form multinucleate fragments or mononucleate cells. In addition, levels of muscle-specific transcripts such as MyoD, myogenin and MRF4, as well as the cyclin-dependent kinase inhibitor p21, which plays a role in maintaining the cell cycle arrest in myotubes, were down-regulated in 30-50% of myotubes. Around 9% of myotubes were induced to undergo cleavage, just over half of these represented cases of cellularisation in which mononucleate progeny were formed and then proliferated, as judged by regular imaging of the culture at 12 or 24 hour time points.

Msx-1 is a homeodomain transcription factor with a previously documented role in differentiation and regeneration. It appears to function as a transcriptional repressor and is expressed in the developing embryo in regions of mesenchymal-epithelial interaction. Expression of Msx-1 appears to function to maintain cells in a less differentiated state, as demonstrated in migration of muscle precursors from the somite during development, where Msx-1 is coexpressed with Pax3 and is thought to negatively regulate Pax3, to delay differentiation during migration (Bendall et al., 1999). Over-expression of Msx-1 in myogenic cells represses MyoD expression and blocks myogenic differentiation (Bendall et al., 1999; Odelberg et al., 2000; Song et al., 1992), and is also reported to antagonise differentiation in other cell types (Hu et al., 2001). Homologues have been identified in a range of species including birds, fish, amphibians and mammals.

During limb regeneration in the urodele amphibians, Msx-1 is present at low levels in both normal and regenerating limbs (Crews et al., 1995) and appears to be up-regulated in the mass of undifferentiated proliferating mesenchymal cells of the blastema (Simon et al., 1995) analogous to its expression in the progress zone at the tip of the developing limb bud during embryogenesis (Koshiba et al., 1998; Song et al., 1992).

In mammals, digit tip regeneration in both humans and mice has been correlated with expression of Msx-1. During limb development, expression becomes increasingly distally restricted and during digit formation is expressed only at the distal tips; by birth expression has also become dorsally restricted, to the region of the nail bed. Regenerative potential appears to be closely associated with the Msx-1 expression domain. Amputation through the region of expression results in regeneration while amputations proximal to the Msx-1 domain fail to undergo successful regeneration, a phenotype also seen in Msx-1 mutant digits (Han et al., 2003; Reginelli et al., 1995). Expression of Msx genes has also been implicated in zebrafish fin regeneration (Akimenko et al., 1995).

1.4.3 The relationship between S phase re-entry and cellularisation

Although the issues of cell cycle re-entry and cellularisation have been dealt with separately here in order to emphasise these two important indices of dedifferentiation, the coordinated reversal of both cell cycle arrest and the mononucleate to multinucleate transition is required for successful dedifferentiation and the formation of proliferating mononucleate cells capable of contributing to regeneration in the urodele limb. The relationship between the two aspects of dedifferentiation remains unclear.

Cellularisation has been shown to occur under conditions where cell cycle re-entry is blocked (Velloso et al., 2000), and in primary axolotl myofibres, cellularisation also appeared to take place within myofibres without prior reversal of the post-mitotic block, S phase re-entry being seen only in the mononucleate progeny (Kumar et al., 2004). This raises the possibility that re-entry into the cell cycle lies downstream of, and is triggered by, cellularisation. In contrast, the timing of cell division in mononucleate progeny of axolotl tail myofibres *in vivo* led the authors to speculate that S phase re-entry was likely to have occurred prior to, or during, the process of cellularisation (Echeverri et al., 2001).

Studies of newt myotubes in culture confirm that entry into S phase is possible in multinucleate myotubes following stimulation with high serum (Tanaka et al., 1997), although it should be noted that these nuclei were then blocked in S/G2 and did not progress into mitosis. So while reversal of the post-mitotic arrest and the multinucleate state may be activated independently by different stimuli, or when one aspect is blocked by experimental manipulation, the interaction between the two during regeneration and the molecular basis of such a process remains open to debate.

Dedifferentiation of myotubes in the mammalian and urodele systems have also been examined separately but the main purpose of studying the two systems is of course to consider whether there are shared aspects and common mechanisms between the two and whether our understanding of dedifferentiation in the urodele context can be applied to mammalian cells. Several lines of evidence from such comparisons provide optimism that we may be able to stimulate mammalian cells to undergo urodele-like regeneration processes, and makes the case that the study of urodele regeneration is relevant to understanding maintenance of the differentiated state in mammalian cells, and manipulating wound healing and the injury response.

Studies have revealed that the post-mitotic arrest in both urodele and mammalian myotubes appears to be mediated by the same molecules, namely the retinoblastoma protein and its binding partners, and a major task now is understanding how these proteins are regulated differently in the two systems. The discovery of a blastema extract capable of inducing both mammalian and newt myotubes to apparently undergo dedifferentiation reveals that regulation between the two may not be so different after all in the presence of the correct signals. Although the factors in the blastema, or in mammalian serum, responsible for triggering aspects of dedifferentiation in myotubes remain unknown, studies in both axolotl (Kumar et al., 2004) and mouse (Odelberg et al., 2000) suggest that Msx-1 may function downstream of these elements and act as a master regulator of both cellularisation and cell cycle re-entry. A further understanding of the role of Msx-1 in urodele regeneration is required but knowing that this potentially pivotal gene is conserved in mammals, and appears to fulfil a similar function in differentiation, provides an intriguing development towards the goal of integrating our understanding of plasticity and regeneration in the two systems.

1.5 CELLULARISATION BY MYOSEVERIN

1.5.1 The molecular and cellular properties of myoseverin

1.5.1.1 Isolation of myoseverin from a combinatorial chemical library

A number of reports describing the reversal of differentiation in both mammalian and urodele myotubes have been detailed above. While some progress has been made towards identifying the molecules responsible for maintaining the cell cycle arrest in myotubes and therefore understanding the mechanism by which the arrest can be over-ridden in response to a number of experimental stimuli, the examples of cellularisation are fewer and far less understood. In recent years, a small chemically synthesised molecule and its derivatives have been reported to bring about cellularisation of mammalian myotubes (Perez et al., 2002; Rosania et al., 2000), and to alter gene expression in a manner indicative of the activation of a regeneration-like response (Rosania et al., 2000); myoseverin, which has been referred to briefly above, will now be described in more detail, and its significance in the context of regeneration research and as the initial motivation for the work contained in this thesis will be discussed.

The utilisation of small molecules to unravel complex biological processes and systems is not a novel concept and in the past scientists have taken inspiration from nature, identifying naturally occurring molecules which interfere with particular biological pathways. The chemical synthesis of these naturally occurring bioactive compounds and the design and modification of synthetic analogues has been a major driving force in the search for novel drug candidates. However, this approach to drug discovery looks set to be superseded by advances in the synthesis and screening of large combinatorial chemical libraries which can be used to identify bioactive compounds without prior knowledge of the protein target (Arya et al., 2001). The discovery of myoseverin provides an excellent example of a rapidly emerging field of research often referred to as 'chemical biology'.

Despite moving away from the traditional target-orientated synthesis of small molecules, the generation of large chemical libraries is still driven by our biological knowledge of the system we wish to manipulate. Many signalling pathways involve protein kinases, and membrane-associated tyrosine kinases are often mutated into a constitutively active form in human cancers, for example Src family kinases are mutated in almost all breast

cancers, and so discovering drugs which can selectively and efficiently inhibit kinases is a major task. Cyclin-dependent kinases (Cdks) are involved in many biological processes and are key regulators of the cell cycle and as such form a significant target for kinase inhibiting drugs. Understanding of the ligands of Cdks has led to the synthesis of libraries based on the purine ring, almost all current kinase inhibitors are ATP-competitive, and interact with the ATP-binding pocket of the proteins (Cohen, 1999). Purines are present in all cells and as well as contributing to the structure of nucleic acids they are bound by a wide range of proteins including GTPases, polymerases and kinases. Previous strategies have used purine analogues to target kinases and they have been shown to make effective Cdk inhibitors. The library of 2,6,9-trisubstituted purines, from which myoseverin was isolated, was originally designed, by Schultz and colleagues, to identify Cdk inhibitors with improved potency and specificity (Chang et al., 1999). Given the ubiquitous nature of such molecules in a cell, a library of molecules based around the purine motif may be expected to yield a high number of bioactive compounds so, in addition to successfully isolating several kinase inhibitors, the library was used in a secondary screen to look for molecules which may alter the differentiation state of mammalian myotubes.

1.5.1.2 The cellular and morphological effects of myoseverin on mammalian myotubes

As previously discussed, regeneration in the urodele involves both cellularisation and cell cycle re-entry in myotubes resulting in the reversal of the terminally differentiated state of muscle cells. The purpose of this secondary screen was to potentially identify a small molecule that could trigger such events in a mammalian context. The substituted purine library was tested on the murine muscle cell line C2C12. The cells were grown and differentiated to form myotubes in 96-well plates to facilitate high throughput screening of the compounds. Myoseverin was initially isolated due to its striking physical effects on the morphology of myotubes, visible simply by observing the cells with bright field microscopy. This compound appeared to fragment the myotubes, causing the multinucleate cells to be broken down into smaller fragments and mononucleate cells. The process proceeded by a narrowing of the cell on either side of the nuclei until only thin cytoplasmic bridges joined the cell, giving the myotube an appearance reminiscent of 'beads on a string' (Rosania et al., 2000) (Figure 1-6). These narrow connections eventually broke down to leave mononucleate and multinucleate fragments with no loss

of membrane integrity. The authors noted that the process seemed to be most efficient on myotubes with a long thin morphology in which nuclei were aligned in a single column along the length of the myotube. Such an arrangement would intuitively appear to be more conducive to the events of cellularisation as described, than a cell shape where nuclei are grouped together in a wider and more rounded myotube.

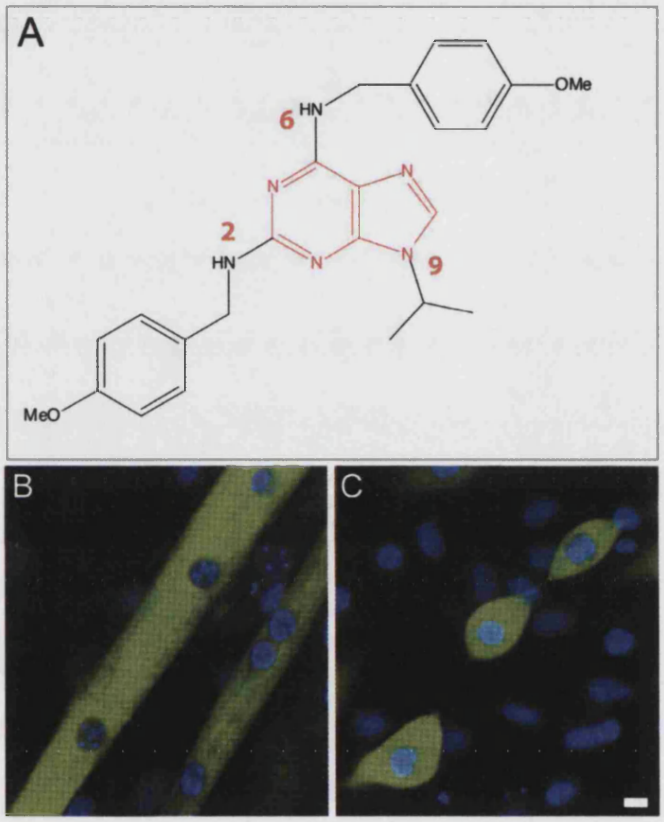


Figure 1.6: Myoseverin is a synthetic molecule which brings about cellularisation of mammalian myotubes. Myoseverin is a 2,6,9-trisubstituted purine isolated from a combinatorial chemical library (A). C2C12 myotubes are shown before (B) and after (C) treatment with 20µm myoseverin, MHC staining is shown in green, nuclei are blue. Constrictions along the length of the myotube gives the cells a 'beads on a string' appearance which resolves to give viable mononucleate progeny. Scale bar = 10µm. (Modified from Rosania et al; 2000)

Although the original report of myoseverin provided no quantitation of its effect on myotubes, giving little indication as to the proportion of myotubes affected, in a subsequent report myotube disassembly was measured by recording the average number of nuclei per myotube at 5-hour timepoints over a 24 hour treatment period with 10µM myoseverin, after staining the cells for myosin heavy chain (MHC) and nuclei. The results showed a gradual drop in the number of nuclei per myotube, from an average of around 30 in control experiments, to a culture containing a predominantly mononucleate population following myoseverin treatment, suggesting a high efficiency of

cellularisation and a widespread effect on all myotubes in the dish. This is in contrast to the cellularisation of mammalian myotubes by ectopic Msx-1 expression (Odelberg et al., 2000), or treatment with a prepared blastema extract (McGann et al., 2001), as described above; in both cases, only around 10% of myotubes underwent a fragmentation event.

Examination of the cytoskeleton in myotubes treated with myoseverin, by immunofluorescence, revealed a dramatic rearrangement of the microtubule cytoskeleton. Following treatment, the long straight microtubules of control cultures were broken into short fragments in the cytoplasm or were clustered around the nuclei. Points of narrowing and ultimately fission of the myotube reportedly corresponded to regions devoid of microtubules. In contrast, organisation of the actin cytoskeleton appeared to be unaffected.

These initial indications that myoseverin was a microtubule-binding molecule were confirmed by addition of a biotinylated form of myoseverin to cells. Cell extracts then mixed with streptavidin beads identified tubulin as binding with myoseverin. In addition, an *in vitro* assay using purified bovine tubulin, showed that myoseverin inhibited microtubule polymerisation, in agreement with the staining pattern and apparent decrease in polymer mass observed by immunofluorescence in treated cells. Thus, myoseverin appears to be a novel microtubule-depolymerising agent.

Comparison of myoseverin with the known microtubule-binding molecules, colchicine, nocodazole, vinblastine and taxol reportedly lead to the contraction of myotubes and formation of 'myosacs' with nocodazole, colchicine and vinblastine, while the action of taxol brought about a fission process reminiscent of the action of myoseverin (Rosania et al., 2000). Nocodazole and colchicine inhibit microtubule polymerisation through binding to the same site on tubulin, while vinblastine also promotes microtubule depolymerisation through an alternative mechanism; taxol is a microtubule-stabilising agent (Hyams and Lloyd, 1994). An extension of this work in a subsequent paper, looking more closely at the effects of myoseverin and its derivatives on myogenic differentiation, showed that all four of the microtubule-binding agents were capable of inducing both microtubule depolymerisation and myotube fragmentation, while in the *in vitro* polymerisation assay, the effect of nocodazole closely resembled that of myoseverin

(Perez et al., 2002). However, the effect of myoseverin was shown to be reversible while that of taxol was not, and examination of cell viability demonstrated that myoseverin was considerably less toxic to cells than the other molecules, producing viable, non-apoptotic cell fragments.

The reversible nature of myoseverin was established by washing out the compound and maintaining the cells in differentiation medium again. Under these conditions, long narrow myotubes reformed and quantitation of the number of myotubes, and the number of nuclei per myotube, over a four day period following myoseverin treatment, revealed that the cells underwent fusion and differentiation resulting in a culture virtually indistinguishable from control untreated cultures. These observations further supported the findings that cellularisation by myoseverin results in the formation of viable mononucleate fragments.

However, perhaps one of the most significant properties of the mononucleate progeny produced by the fragmentation of myotubes with myoseverin is not the ability of these cells to fuse back to form myotubes again, but their ability, when stimulated with high serum growth medium to apparently undergo S phase re-entry and proliferation. Following myoseverin treatment, the cells were incubated with bromodeoxyuridine (BrdU) and growth medium. Overall incorporation of BrdU into the culture, indicating entry into S phase, was measured using an ELISA type assay based on absorbance measurements in a plate reader. The results indicated that myoseverin stimulated cell proliferation within the culture in a serum-dependent manner, compared to untreated cultures, suggesting that myoseverin was capable of reversing not only the mononucleate to multinucleate transition in myotubes, but also the post-mitotic block.

The structure of myoseverin is shown in Figure 1-6. Structure-activity relationship studies on myoseverin revealed that the two methoxy-benzene groups at positions C2 and C6 were essential for tubulin depolymerisation and for myotube fragmentation activity. Removal, substitution or even repositioning of the methoxy groups around the benzene ring completely abolished activity (Perez et al., 2002; Rosania et al., 2000). Greater diversity was possible at the N9 position with hydrophobic moieties often increasing the activity, as did increased chain length (Perez). Ethyl and methyl groups showed a

progressive decrease in activity compared to the isopropyl group of myoseverin (Rosania et al., 2000).

1.5.1.3 Changes to gene transcription in response to myoseverin

In addition to the morphological changes brought about by myoseverin, a second major aspect to its action on mammalian myotubes was revealed by an accompanying microarray analysis. Examination of the gene expression profile showed that the cellular effects of myoseverin, reminiscent of the cellularisation events that take place during urodele limb regeneration, appeared to be complemented by a transcriptional programme also indicative of regeneration-like processes.

Differentiated cultures were treated for 24 hours with either 20μM myoseverin or a structurally similar but inactive derivative. Using a microarray approach, the expression of over 6000 transcripts were analysed for changes over the 24 hour treatment period. The results were striking; of 93 genes altered significantly by myoseverin, over 50% were deemed to be involved in processes suggestive of a cellular response to tissue injury.

These included genes involved in extracellular matrix (ECM) remodelling, growth factors and growth factor inducible genes. Also altered were genes involved in inflammation, coagulation, signal transduction and several genes with a role in the recruitment of cells of the immune system. Processes such as protein turnover and response to oxidative stress were up-regulated while apoptosis appeared to be down-regulated. Cytoskeletal genes were generally down-regulated, consistent with disruption of the microtubule network, while genes involved in myogenesis, metabolism and the cell cycle were largely unaffected.

The role of the ECM and remodelling enzymes has been described during regeneration of various tissues (Goetsch et al., 2003; Michalopoulos and DeFrances, 1997) and the breakdown and deposition of ECM components appears to play a role during urodele limb regeneration (Tsonis, 1996). The coagulation cascade has also been implicated during urodele regeneration, through the discovery of thrombin activation at specific sites following limb amputation (Tanaka et al., 1999) and lentectomy (Imokawa and Brockes, 2003) in the newt. Overall, many of the processes which appear to be regulated in

response to myoseverin are consistent with a role for this molecule in wound healing and regeneration. An analogy has been drawn between the results obtained here with myoseverin and a similar microarray approach to analysing the serum response of fibroblasts (Iyer et al., 1999).

The serum response of quiescent fibroblasts in culture consists of re-entry into the cell cycle and rapid proliferation, processes that have been studied extensively using these cells as a model. Many genes which play a role in this activation and proliferation have been identified and it was felt that the response of fibroblasts to serum was a well-characterised process. However, the results of a microarray study of the response revealed what investigators had missed during years of studying the process; although growth and proliferation were activated, a more extensive programme indicative of a cellular response to injury was also seen (Iyer et al., 1999). Fibroblasts play a significant role during the early response to wounding, participating in clotting and fibrosis, chemotaxis and angiogenesis. The microarray study concluded that what had previously been thought of as a general mitogenic response to growth factors in the serum, was actually a more complex and physiologically relevant response of fibroblasts to a stimulus normally encountered in the context of wounding.

Although myoseverin is a synthetic molecule, the response of a differentiated myotube culture to myoseverin seems to involve many of the same processes as the fibroblast response to serum, which describes the type of physiological response activated during tissue injury. In both cases, the application of microarray technology revealed a greater depth of understanding than observations of cellular behaviour and morphological changes alone could provide.

1.5.1.4 Molecular dedifferentiation of myogenic cultures by myoseverin

In order to further characterise the molecular events of apparent dedifferentiation by myoseverin, a number of muscle-specific transcription factors, as well as MHC and molecules involved in cell cycle progression were analysed by immunoblotting (Perez et al., 2002). During myoseverin treatment of differentiated cultures an increase in MHC, MyoD and Myf5 was seen, following removal of myoseverin and incubation in growth medium, levels of these proteins fell, with an accompanying increase in levels of cyclin

A and Cdk2 which are associated with the progression from G1 to S phase in cycling cells. In proliferating mononucleate myoblast cultures, myoseverin has been shown to inhibit growth and differentiation as judged by inhibition of myotube formation in the presence of the compound (Perez et al., 2002), further supporting a role for myoseverin in mediating myogenic differentiation.

While the mechanism of action of myoseverin is not understood, the depolymerisation of microtubules indicates a possible means by which myotube disassembly can occur. Although the microarray analysis does not show direct activation of cell cycle machinery, the authors speculate that re-entry into S phase and the ability to proliferate may be restored through the release of inhibitory mechanisms brought about by re-establishing the mononucleate status of the cell (Rosania et al., 2000).

It should be noted that although myoseverin was isolated from a library originally designed to isolate kinase inhibitors, it did not show any activity against Cdk1 despite sharing the purine ring motif with other molecules, isolated from the same library, in the original screen for Cdk inhibitors. Although both myoseverin and the purine-based kinase inhibitors were shown to inhibit cell cycle progression, the Cdk inhibitors did not depolymerise microtubules, clearly establishing that despite their common origin, myoseverin acts through an entirely different mechanism, which is not mediated through kinase inhibition (Chang et al., 2001).

1.5.2 The role of microtubules in myotube morphology and differentiation

As microtubules are the only known target of myoseverin, and depolymerisation accompanies the cellularisation of myotubes, the organisation of the microtubule cytoskeleton in the target cell should be considered in a discussion of the mechanism of action of myoseverin. Microtubules have been shown to play an important role in driving changes in cell shape and in acquisition and maintenance of a differentiated phenotype in cells where the differentiation and function of a cell requires changes in morphology (Bulinski and Gundersen, 1991; Vega and Solomon, 1997). The process of myogenesis, whereby mononucleate cells fuse to form multinucleate myotubes has been described previously, and involves the elongation, alignment and fusion of individual myoblasts. Changes to the organisation of the microtubule cytoskeleton during

myogenesis have been well documented. In mononucleate myoblasts, as in many animal cells, the microtubules radiate outwards towards the cell cortex from a single point, close to the nucleus. During myogenesis, this radial organisation is replaced by a linear array, rather than nucleation from a single point, microtubules run in parallel, aligned with the long axis of the elongated myotube.

The mononucleate cells begin to elongate prior to fusion by a process in which microtubules extend into the growing tips of the cell (Saitoh et al., 1988) and if treated with nocodazole the cells retract. Several studies describe rounding up of myotubes and formation of 'myosacs' upon treatment with microtubule-disrupting agents (Bischoff and Holtzer, 1968; Saitoh et al., 1988) highlighting the importance of microtubules in cell shape. In addition to maintaining the morphology of differentiated myotubes, microtubules may also have a further role in differentiation during myofibrillogenesis. It has been suggested that microtubules may act as a scaffold to guide myofibrils, and interference with microtubule organisation has been shown to cause severe disruption of myofibrillogenesis (Antin et al., 1981; Toyama et al., 1982).

In most mammalian cells, microtubules are nucleated from a distinct cellular MTOC (microtubule-organising centre), the centrosome. Each cell contains a single centrosome, comprised of two centrioles and the surrounding PCM (pericentriolar matrix), a meshwork containing proteins required for microtubule nucleation. One of the best characterised of these, and a constitutive component of the centrosome is gamma-tubulin, which forms ring complexes that nucleate the microtubules. While centrioles do not participate directly in microtubule nucleation they act to focus and localise the PCM.

During myogenesis, the centrosome ceases to function as the major MTOC of the cell, concomitant with reorganisation of the microtubule cytoskeleton from a radial to a linear array. Prior to and during fusion, centrioles are still detected in the myoblasts but after fusion, these distinctive organelles appear to be lost from mature myotubes. The loss of centrioles has been documented in early electron microscopy studies (Przybylski, 1971; Warren, 1974) and by immunofluorescence using antisera to centrosomes (Connolly et al., 1986; Tassin et al., 1985) and more recently to the specific centriolar proteins γ-tubulin and pericentrin (Musa et al., 2003). The centrioles are progressively lost and are

reportedly completely absent from mature muscle fibres (Przybylski, 1971). In mononucleate cells, the centrosome and its radial array of microtubules functions to enable polarity, migration and cell division. In the myotube, these activities are no longer needed and the requirements of the cell are better served by a reorganised linear array.

While a radial microtubule arrangement is required for cell motility and division, a non-centrosomal array enables the cell to realise a wider range of cell shapes and the myotube is not unique is utilising such a mechanism; other specialised cell types, most notably neurons and polarised epithelium possess non-radial microtubule arrays. The means of establishing such arrays is unclear although a number of possible mechanisms, including release from the centrosome, cytoplasmic nucleation or breakage of microtubules, have been proposed (Keating and Borisy, 1999). During the development of epithelial polarity, the centrosomes continue to nucleate microtubules, but these newly formed microtubules are then released from the PCM and anchored at alternative sites to establish a microtubule array in which the minus ends lie apically, and the plus ends at the basal end (Bacallao et al., 1989; Mogensen et al., 2000). In neurons, the outgrowth of the axon also appears to proceed by centrosomal nucleation of microtubules, followed by release. In this case, the microtubules are then transported along the axon by molecular motors (Baas, 1998).

The consequences of such a specialised microtubule array for the reversal of the differentiated state of myotubes, and the role of microtubules in the mechanism of action of myoseverin and in dedifferentiation, will be discussed in more detail in subsequent chapters of this thesis.

1.5.3 Chemical biology

The use of small molecules to direct stem cell fate is not a new idea. 5-azacytidine, a small molecule DNA demethylating agent, induces myogenic differentiation of fibroblast cell lines and proved a valuable tool in the discovery of MyoD as a master regulatory gene of myogenesis (Lassar et al., 1986). Dexamethasone has been shown to induce a number of different phenotypes in cultured cells including chondrogenesis and adipogenesis (Grigoriadis et al., 1988), and numerous other natural molecules, such as

retinoids and ascorbic acid have been used to differentiate a range of pluripotent progenitor cell lines (Ding and Schultz, 2004).

When grown in suspension, embryonic stem cells (ESCs) will form embryoid bodies which undergo spontaneous differentiation giving rise a variety of cell types. But this method yields an undefined population of differentiated and undifferentiated cells, and a low frequency of any one specific cell type. The current interest in the potential of cultured ESCs to act as therapeutics, or activation of a patients own resident stem cell populations to repair damaged tissue, is accompanied by a growing need to discover the pathways involved in differentiation and to identify protocols to enable the efficient and directed differentiation of multipotent cells into functional mature cell types *in vitro*. The use of retinoic acid (RA) to form motor neurons has been described, but in addition to initial treatment with RA, which promoted neural differentiation, the cells then required a second signal in the form of Sonic hedgehog to differentiate fully into motor neurons (Wichterle et al., 2002). In this case the authors were applying knowledge from *in vivo* studies of the differentiation of motor neurons, which highlights the fact that differentiation often requires multiple steps leading to the development of elaborate cocktails of growth factors and signalling molecules for differentiation in culture. In many cases however, details of the differentiation pathway are unknown and the factors involved only poorly characterised.

In recent years, the approach used to identify myoseverin has been utilised with much success to identify molecules that can direct the differentiated state of ESCs. Instead of using knowledge of the developmental pathway of interest to direct the design of a differentiation strategy, chemical biology can perhaps be thought of as taking the reverse approach, requiring only a basic idea of the types of molecules likely to be involved. This method begins with tens of thousands of chemically distinct molecules and by identifying active compounds, then determining their cellular target, can provide insight into the endogenous proteins involved in differentiation. The structures of some of the active small molecules described below are shown in Figure 1-7. A 4,6-disubstituted pyrrolopyrimidine, designated TWS121, was isolated in a screen for compounds displaying the ability to induce neural differentiation in an embryonic stem cell line. The cellular target of this molecule was found to be GSK-3β, a multifunctional kinase implicated in cell fate determination and downstream of Wnt signalling, in agreement

with evidence that antagonists of Wnt signalling or a dominant negative GSK-3β can induce neuralisation of ESCs (Ding et al., 2003). Such an example demonstrates the value of chemical biology as a tool in the elucidation of molecular mechanisms of biological processes, as well as its more applied role in drug discovery.

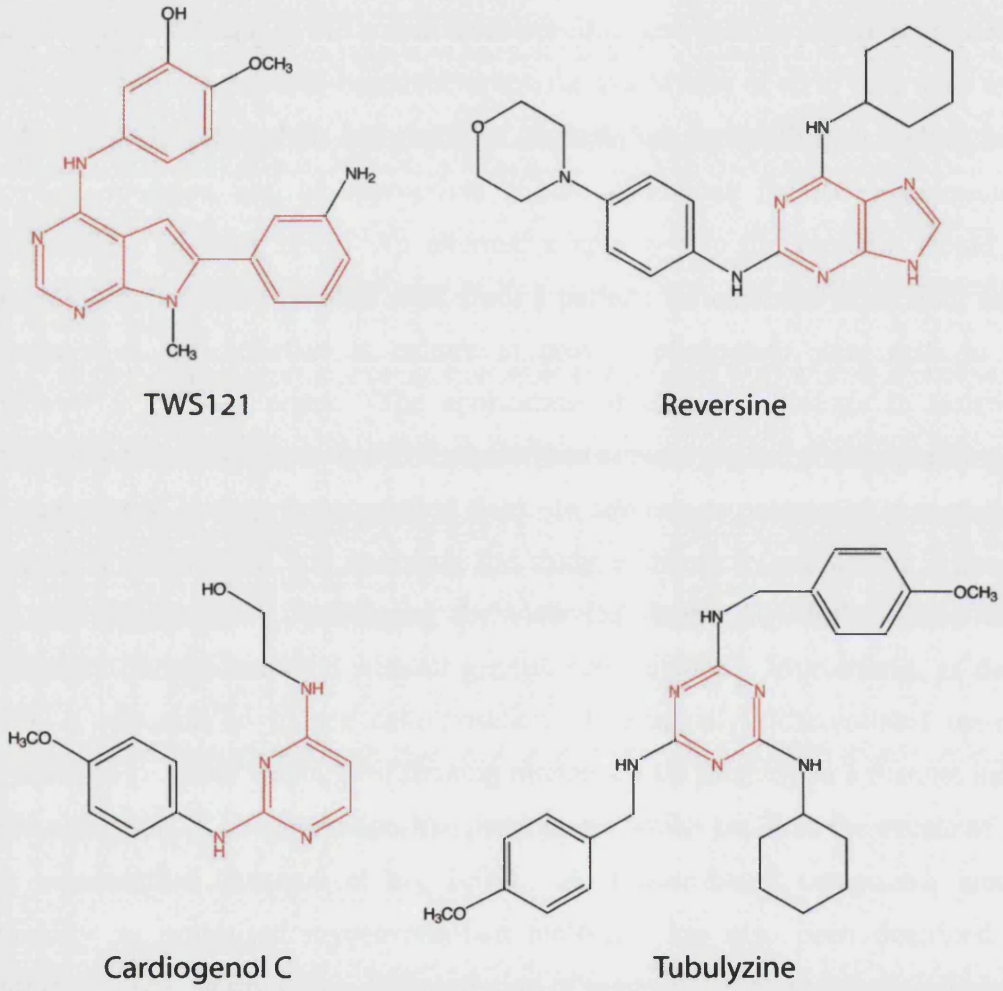


Figure 1-7: Structures of synthetic small molecules that regulate cell fate. All four compounds shown here were isolated by large scale phenotypic cell-based screening of combinatorial chemical libraries of heterocyclic compounds. TWS121 (a 4,6-disubstituted pyrrolopyrimidine) induces osteogenesis in multipotent mesenchymal progenitor cells; cardiogenol C (a diaminopyrimidine) induces formation of cardiomyocytes from multipotent stem cells. Tubulyzine (a 2,6-disubstituted triazine) and reversine (a 2,6-disubstituted purine) act on post-mitotic multinucleate myotubes and committed mononucleate myoblasts respectively, to induce dedifferentiation. For each compound, the scaffold used for synthesis is highlighted in red.

Other examples of molecules recently isolated from large combinatorial libraries include purmorphamine, a compound with osteogenesis-inducing activity (Wu et al., 2002), and cardiogenol C with cardiomyogenic properties (Wu et al., 2004). The heart contains a

population of cells that may re-enter the cell cycle in response to damage, but their regenerative capacity appears to be limited; treatment of ESCs with cardiogenol C generated cultures of beating cardiac muscle expressing appropriate markers (Wu et al., 2004).

While the manipulation of ESCs and tissue-specific stem cells by small molecules offers an attractive approach to cell-based therapies, the availability of stem cells from embryos and from certain areas of the body such as the brain can be limiting, as well as issues of transplant rejection and of appropriate culture conditions for the maintenance and differentiation of these cells. An alternative approach to the problem would be the manipulation in culture of adult cells from a patient, for example from skin, and their subsequent dedifferentiation in culture to provide pluripotent stem cells to treat a deficiency in another organ. The application of chemical biology to isolate small molecules which can induce dedifferentiation has already yielded some promising results and looks set to become an expanding field. In addition to potentially providing useful therapeutics, such work will stimulate the study of plasticity within the differentiated state, and regeneration, challenging the view that terminally differentiated cells are irreversibly lineage-restricted without genetic manipulation. Myoseverin, as described above, is reported to induce cellularisation of terminally differentiated mammalian myotubes to generate viable, proliferating mononucleate progeny in a manner indicative of the activation of a regeneration-like programme which parallels the events of urodele limb regeneration (Rosania et al., 2000). A triazine-based compound, tubulyzine, apparently an optimised myoseverin-like molecule, has also been described and in addition to blocking myogenic differentiation of mononucleate myoblasts, improves their survival when implanted into a mouse model of muscular dystrophy (El Fahime et al., 2003).

A further report describes reversine, which, like myoseverin, is a substituted purine, and was isolated in the same lab (Chen et al., 2004). Reversine treatment induced reversal of lineage commitment in C2C12 cells, a muscle cell line, resulting in formation of multipotent mesenchymal progenitor cells capable of osteogenic, adipogenic or myogenic differentiation under the appropriate culture conditions. In the presence of reversine, mononucleate C2C12 cells failed to activate their typical programme of

myogenesis in culture consisting of withdrawal from the cell cycle and fusion to form multinucleate myotubes, and continued to proliferate, while levels of muscle-specific markers decreased. The cellular target of reversine remains to be elucidated.

1.6 FOCUS OF EXPERIMENTS FOR THIS THESIS

Myogenic differentiation involves withdrawal from the cell cycle by lineage committed myoblasts, followed by fusion to form post-mitotic multinucleate myotubes. Cell cycle withdrawal and the mononucleate to multinucleate transition provide two indices of the differentiation process, and make skeletal muscle an ideal model system for the study of differentiation events. During urodele limb regeneration, differentiated cell types at the site of amputation undergo dedifferentiation and contribute to the mass of proliferating cells, known as the blastema, and ultimately to the tissues of the regenerate, demonstrating a remarkable degree of plasticity of the differentiated state. For skeletal muscle, dedifferentation involves not only re-entry into the cell cycle but also cellularisation resulting in the formation of mononucleate cells from the multinucleate syncytium of the myotube.

Recent work has suggested that mammalian cells may possess a greater capacity for plasticity of the differentiated state than previously thought and has given impetus to the study of dedifferentiation and regeneration. The search for means by which to induce regeneration-like processes in mammalian cells has generated a number of reports, one of which described myoseverin, a small purine-based molecule which was reported to bring about cellularisation of mammalian myotubes and cell cycle re-entry in the resulting mononucleate cells to generate proliferating mononucleate cells.

The initial motivation for the work in this thesis was the potential for inducing a programme in mammalian cells characteristic of that involved in urodele regeneration. The isolation of myoseverin from a combinatorial chemical library has provided the starting point for the work described here and a major focus of the experiments contained in this thesis has been study of the mechanism of action of myoseverin, and evaluation of the hypothesis that myoseverin is activating a regeneration-like programme in mammalian cells.

Although cellularisation of both urodele and mammalian myotubes has been documented under a range of experimental conditions, the cellular and molecular basis of cellularisation is poorly understood so I have used myoseverin and its derivatives to probe the mechanism of cellularisation, and have tried to define a role for this aspect of the dedifferentiation process within a wider programme of regeneration.

The work carried out during the first year of my PhD was done using the original murine C2C12 cell line, which was later abandoned after extensive efforts to develop an efficient assay failed to produce a workable and reproducible system. Although experiments with C2C12 cells are mentioned briefly in Chapter 3, the main body of work described in this thesis was done using the Pmi28 cell line, also a murine muscle cell line, which I worked with for the remainder of my PhD. Using Pmi28 myotubes I subsequently demonstrated cellularisation at a single cell level, and focussed on the role of microtubules, the only known target of myoseverin, in bringing about cellularisation of multinucleate myotubes. The application of microarray technology allowed me to study the process of cellularisation at a molecular level, and time-lapse microscopy has been invaluable for observing the cellular and morphological effects of myoseverin on mammalian myotubes. During the final year, my work focussed on determining the consequences of cellularisation for the resulting mononucleate progeny.

Chapter 2

Materials and Methods

2.1 CELL CULTURE

2.1.1 Cell lines

2.1.1.1 C2C12

Cells were grown on plastic 75cm^2 flasks, in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 20% foetal calf serum (FCS; First Link), 0.29 mg/ml glutamine (Gibco), 0.28 i.u./ml insulin (Sigma), 50 i.u./ml penicillin and 50 μg/ml streptomycin, in a humidified atmosphere with 10% CO_2 at 37°C. Cells were passaged at a 1:10 ratio every 2-3 days ensuring that a low density was maintained to prevent contact between cells. For differentiation, cells were plated onto laminin-coated dishes or 12-well plates and grown to >90% confluence, then maintained in a differentiation medium containing 0.5% horse serum (Sigma) in place of 20% FCS for 4 days. Medium was changed after 24 hours and 72 hours. The C2C12 cells used here were a subclone (D12) showing high fusogenicity on myogenic differentiation, derived in this lab by Andras Simon.

2.1.1.2 Pmi28

Pmi28 cells were grown on BIOCOAT collagen I T-75 flasks (Beckton-Dickinson) in nutrient mixture F-10 (HAM) supplemented with 20% FCS (Sigma, SLI Ltd), 50 i.u./ml penicillin and 50 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37°C. Cells were passaged at a 1:15 ratio every 3 days to maintain a low cell density. For differentiation, cells were plated at high density onto collagen-coated plastic dishes or silane-coated glass coverslips (in 4-well plates) and maintained in DMEM containing 10% horse serum, 50 i.u./ml penicillin and 50 μg/ml streptomycin for 3 days, medium was changed after 48 hours.

As cell properties and fusogenicity of myoblasts can alter with prolonged culture, Pmi28 cells were always discarded after 25 passages.

2.1.1.3 LiSa-2

Cells were grown on plastic 75cm^2 tissue culture flasks in Preadipocyte Growth Medium consisting of Preadipocyte Basal Medium supplemented with 10% FCS, 2mM L-glutamine, 100 units/ml pen/strep (Cambrex). Growth medium was changed every 3

days and cells were passaged at a 1:3 ratio every 6-7 days. At >90% confluence cells were transferred into Adipocyte Differentiation Medium consisting of Preadipocyte Growth Medium supplemented with 10μg/ml insulin, 1μM dexamethasone, 200μM indomethacin and 500μM isobutylmethylxanthine, for 15 days, medium was changed every 3-4 days.

2.1.2 Trypsinisation and passaging

Trypsin (Gibco) was diluted from a 10X stock solution of 2.5% in Dulbeccos phosphate buffered saline (D-PBS). Cells were washed once with D-PBS then incubated with 1X trypsin, 2-3ml per 75cm^2 flask. Addition of 4ml of growth medium containing FCS inactivated the trypsin and the cell suspension was centrifuged at 1000rpm (180 x g) for 3 minutes. The pellet was resuspended in 5-10ml of growth medium for dilution into sterile tissue culture flasks.

2.1.3 Coating dishes and coverslips for cell culture

All coverslips were 13mm diameter, no. 1 thickness glass coverslips (BDH) and were cleaned prior to coating as follows. Coverslips were soaked for one hour in concentrated nitric acid, then rinsed three times in distilled water followed by three times in 100% ethanol. Plastic dishes (Nunc) were untreated prior to coating.

2.1.3.1 Collagen

A 0.15% stock solution of collagen type V (Sigma) was prepared in distilled water, autoclaving the solution to dissolve the collagen, followed by filtering through a 0.22μm filter and autoclaving a second time. Culture dishes were coated with enough collagen solution to cover the surface of the dish, after removing the excess, the dishes were allowed to air-dry at room temperature before plating the cells.

2.1.3.2 Laminin

Aliquots of laminin (Sigma) were defrosted on ice then diluted 1:60 in DMEM. Enough volume to cover the surface was added to each sterile culture dish and incubated at room temperature for one hour. Excess liquid was then removed and cells plated immediately.

2.1.3.3 Silane

Coverslips were coated in 0.3% silane (3-(trimethoxysilyl)propyl methacrylate; Fluka), 10% ethanol, 0.3% glacial acetic acid for 30 minutes at room temperature on a rotating shaker. Coverslips were then washed twice for 30 minutes in 10% ethanol, rinsed in 100% ethanol and dried under vacuum for 30 minutes.

2.1.3.4 Matrigel

50µl frozen aliquots of matrigel (Sigma) were thawed at 4°C overnight then diluted into 5ml DMEM previously chilled on ice. 35mm glass-bottomed dishes (Matek) and silane-coated coverslips (see above) were cooled prior to coating and cold pipette tips were used for all manipulations. Diluted matrigel was added to dishes and coverslips in sufficient volume to evenly cover the entire surface. Coated vessels were then incubated at room temperature for 30-60 minutes before removing excess liquid and rinsing briefly in serum-free medium prior to plating cells.

2.1.4 Purification of myotubes

Pmi28 myoblasts were plated onto collagen-coated 60mm plastic dishes which had been scored, prior to coating, with a scalpel to produce a grid of ~1mm squares covering the bottom of the dish. The cells were grown to high density and induced to undergo fusion in differentiation medium for 3 days, the grid on the dish physically limited the size of myotubes that could form. The cells were washed once with D-PBS then trypsinised with 1.5ml 1X trypsin for 3 minutes at 37°C with occasional gentle tapping of the dish to loosen the cells. 3ml of differentiation medium was then added to neutralise the trypsin. Large clumps of cells were gently triturated to dissociate the cells. The cell suspension was passed first through a 25µm filter (BDH) over a 20ml universal tube to remove mononucleates, and the flow-through discarded. Cells retained on the filter were washed from the filter with differentiation medium, into a culture dish and then passed through a 100µm filter (BDH). Cells in the flow-through were plated onto collagen-coated plastic dishes, or matrigel-coated glass coverslips or glass-bottomed dishes.

2.1.5 Transfection

Pmi28 mononucleates were plated onto a collagen-coated 35mm plastic culture dish and grown to approximately 60% confluence. The cells were transfected over night with a

pseudotyped retroviral vector carrying an eGFP reporter construct with a nuclear localisation signal to target the eGFP protein to the nucleus (Yee 1994). Transfection was done in 1ml of growth medium containing 5μg polybrene and was carried out by Anoop Kumar in this lab. Following over night incubation the medium around the cells was changed and the dish was incubated in fresh growth medium for a further 24 hours. Cells were then gently trypsinised and plated into a 75cm^2 culture flask.

2.1.6 PI-PLC treatment

Pmi28 mononucleates on silane-coated coverslips were treated with PI-PLC (Glyko) at a final concentration of 2U/ml in growth medium for one hour at 37°C. The cells were then stained for Sca-1 as described.

2.1.7 Transdifferentiation studies

C2C12 and Pmi28 myoblasts were grown to ~80% confluency in 35mm tissue culture dishes. Cells were washed with D-PBS and then cultured in Adipocyte Differentiation Medium under conditions used for adipogenic differentiation of LiSa-2 cells (see section 2.1.1.3).

2.2 CELLULARISATION ASSAY

2.2.1 Carrying out the assay

For all assay counts with C2C12 cells, the cells were grown on laminin-coated 35mm dishes or 12-well plates. Cells were differentiated for four days in C2C12 differentiation medium before addition of compounds. Between five and seven random fields were scored for each condition/treatment within an experiment and the average recorded.

For assay counts using Pmi28 cells, the cells were plated at high density on silane-coated glass coverslips and differentiated for three days in Pmi28 differentiation medium before addition of compounds. For each condition an average of counts from at least four, but more often six, random fields were taken.

For both C2C12 and Pmi28 cells, all compounds and chemicals were diluted in the cell line-specific differentiation medium unless otherwise stated. For each independent

experiment, duplicate dishes or coverslips were used for every treatment. At the end of the treatment period all cells were fixed and stained with an antibody against MHC and propidium iodide. Mononucleates were defined as cells containing a single propidium iodide stained nucleus, the number of MHC positive cells were counted within a given area and expressed as a percentage of the total number of mononucleates within that area. No discrimination between different levels of MHC expression was made; all cells with detectable MHC were counted as positive.

2.2.2 Assay compounds

2.2.2.1 Myoseverin and derivatives

All purine and triazine compounds were stored at 4°C as 20mM stock solution in DMSO except for myoseverin which was stored as a 25mM stock solution. Compounds were synthesised by Young-Tae Chang (New York University)

2.2.2.2 Microtubule-disrupting agents

Nocodazole (Calbiochem) was made up in DMSO as a 1mg/ml stock solution (3.32mM) and paclitaxel (semi-synthetic; Calbiochem) in DMSO as a 10mM stock. All compounds were diluted in differentiation medium before adding to cells.

2.2.2.3 Protein synthesis inhibitors

Anisomycin (Fluka) was dissolved in distilled water to give a 10mM stock solution. Cycloheximide (Fluka) was dissolved first in a small volume of DMSO to aid solubility then made up to a 10mM stock solution in distilled water. Both compounds were added to culture medium at a final concentration of 1-10μM.

2.3 AUTORADIOGRAPHY

Pmi28 cells were plated onto collagen-coated 35mm dishes and allowed to differentiate for 3 days. Cells were then incubated with tritiated leucine (L-[4,5-^{3}H(N)]-leucine, specific activity 1.6TBq/mmol; NEN Life Science Products), added to the medium at a final concentration of 10μCi/ml for 6 hours in the presence or absence of anisomycin or cycloheximide, then washed three times with fresh medium and once with D-PBS. After washing, cells were fixed with 2% PFA, 2 mins, followed by 100% cold methanol for 5

mins then rinsed with distilled water and allowed to air-dry at room temperature. Cells were coated with Ilford K5 emulsion, melted at 50°C then diluted 1:2 with distilled water, and stored in the dark for an exposure time of 24 hours. Dishes were developed with Ilford Phenisol, diluted 1:5 in distilled water, for 6 mins, rinsed with distilled water and fixed with Ilford hypan, diluted 1:5 in distilled water, for 6 mins. Dishes were washed again with distilled water before quantitation of grain density by light microscopy.

To quantitate incorporation of labelled leucine, developed silver grains in the emulsion were counted as a measure of protein synthesis. At each concentration of protein synthesis inhibitor, the grains directly above myotubes were counted in ten randomly chosen fields. A background level was calculated by counting grains in a dish without cells, but processed in parallel as above.

2.4 IMMUNOCYTOCHEMISTRY

2.4.1 Primary antibodies

All antibody incubations and washes were carried out in D-PBS containing 10% goat serum (Sigma) and 0.05% sodium azide unless otherwise stated. Antibodies against β-tubulin (clone TUB 2.1) and γ-tubulin (clone GTU-88), were mouse monoclonal, IgG1 isotype, from Sigma. BrdU (BU-20, IgG1 isotype; Amersham) and MHC (A4.1025, IgG2a isotype; Dr. Simon Hughes, Randall Institute, Kings College, London) antibodies were also mouse monoclonals. Rabbit polyclonal antibody against pericentrin was purchased from Covance; rat anti-mouse Ly6A/E (Sca-1) monoclonal antibody and (FITC)-conjugated rat anti-mouse TSA-1 (Sca-2, Ly6E) monoclonal antibody were both from Pharmingen.

2.4.2 Fixation and staining

2.4.2.1 MHC

Cells were washed briefly in D-PBS, fixed for 3 minutes in 2% paraformaldehyde (PFA; Sigma), then washed 3 times in D-PBS. Post-fixation was carried out with cold 100% methanol for 5 minutes, followed by two 5 minute washes in D-PBS. Fixed cells were incubated with a monoclonal antibody to mouse MHC, diluted 1:500, for 1 hour on a

rocker, followed by four 10 minute washes. The secondary antibody was diluted 1:100 and incubated with the cells for 1 hour followed by four 10 minute washes and nuclear staining.

2.4.2.2 BrdU

Cells were fixed in 2% PFA, followed by 100% methanol as for MHC staining, then treated for 10 minutes with 1M hydrochloric acid, to partially hydrolyse the DNA and expose the antigen. Staining with a monoclonal antibody to BrdU was carried out overnight at 4°C, with the antibody diluted 1:500. Following four 10 minute washes, cells were incubated with goat anti-mouse IgG1 conjugated with Texas Red (Southern Biotechnology Associates Inc), at 1:100 for 1 hour. Cells were then washed and nuclei stained with Hoechst 33528.

2.4.2.3 γ-tubulin and pericentrin

All buffers and fixatives were filtered through 0.2μm sterile syringe filters, and antibodies were centrifuged at top speed (20 800 rcf) in a bench-top centrifuge for 10 minutes prior to use, in order to remove particulate matter which may compromise centriole detection following staining. Cells were washed once, for 30 secs, in extraction buffer then fixed for 10 minutes on ice, in 4% PFA in 1X extraction buffer. Cells were then washed twice for 5 minutes in extraction buffer + 0.2% Triton X-100, and fixed for a further 5 minutes with cold 100% methanol, followed by two washes in extraction buffer + 0.2% Triton X-100. The cells were incubated with the two primary antibodies, against pericentrin and gamma-tubulin, simultaneously, overnight at 4°C and then washed four times for 15 minutes, followed by incubation with FITC-conjugated swine anti-rabbit, at 1:100, for 1 hour (Dako), three washes, and then Texas Red-conjugated goat anti-mouse IgG1, at 1:100 for 1 hour (Southern Biotechnology Associates Inc). Cells were washed thoroughly and the nuclei stained with Hoechst 33528.

2.4.2.4 β-tubulin

Cells were washed once with D-PBS, then fixed for 10 minutes on ice, in cold 100% methanol, followed by two further washes in D-PBS. Incubation with primary antibody, at 1:500, was carried out overnight at 4°C, followed by four 15 minute washes, and incubation with FITC-conjugated rabbit anti-mouse (Dako).

2.4.2.5 Sca-1 and Sca-2

All staining was carried out on live cells. Cells were incubated with primary antibody, diluted 1:500 in Pmi28 differentiation medium (DM), for 20 minutes, on ice. Cells were then briefly washed four times with cold DM and incubated with fluorescein-conjugated goat anti-rat (Pierce), diluted 1:100, also in DM, plus Hoechst 33342 (Sigma) at 1μg/ml, for 20 minutes, on ice. Following antibody staining, cells were again washed briefly four times with cold DM and once with D-PBS, then fixed for 90 seconds with 5% glacial acetic acid:95% ethanol, made up the same day and chilled at -20°C prior to use.

2.4.2.6 Actin

The phallotoxin, phalloidin, binds specifically to F-actin and was used to detect actin. Cells were washed once in 1X extraction buffer + 0.2% Triton X-100, for 30 seconds, then fixed, on ice, for 10 minutes in 4% PFA made up in 1X extraction buffer, followed by 3-4 washes with D-PBS + 0.1% Tween-20. 5μl or 50μl aliquots of Alexa fluor 488 phalloidin methanolic stock solution (Molecular Probes), prepared and stored according to the manufacturers instructions, were vacuum-dried and reconstituted in 200μl or 2ml D-PBS + 10% goat serum, then incubated with cells for one hour at room temperature, followed by 3-4 washes.

2.4.2.7 Lipid

Cells were washed once in D-PBS, then fixed for 10 minutes in 10% formaldehyde, made up in D-PBS. Nile Red (Nile Blue A Oxazone; Sigma) was dissolved in acetone to produce a saturated solution which was stored, protected from light, at -20°C. Nile Red was diluted 1:100 in 50% glycerol, in D-PBS, and incubated with cells for 5 minutes, the cells were protected from light throughout the staining protocol. Following staining, cells were washed twice with 50% glycerol in D-PBS, twice with 25% glycerol in D-PBS and twice with D-PBS alone, then stained with Hoechst 33258.

2.4.2.8 Nucleic acids

Nuclei of fixed cells were stained with either Hoechst 33258 or propidium iodide (both from Sigma), at a final concentration of 1μg/ml for 3-5 minutes, followed by 3-4 washes in D-PBS.

2.5 MICROARRAYS

2.5.1 Experimental design

Pmi28 cells were plated at high density on collagen I coated 10cm culture dishes (BD Biosciences) and induced to differentiate for three days before treatment with 15μM 109, 500nM nocodazole or 15μM 401 for a range of timepoints. Triplicate cultures were prepared for time zero and for 12 and 24 hour timepoints with 109, in addition single cultures were prepared for 4, 8 and 16 hour timepoints. Triplicate cultures for 12 and 24 hours treatments with nocodazole were prepared, and duplicate cultures for 24 hour 401 treatment. Each individual culture gave rise to material for hybridisation to a single Affymetrix GeneChip, all replicates were treated as independent samples. A total of 20 culture dishes were treated and processed in two separate batches over the course of a month, neither batch contained all three replicates for a given sample point, providing an added level of control for similarities in gene expression arising as an experimental artefact caused by laboratory procedures carried out at the same time.

2.5.2 Sample preparation

2.5.2.1 RNA extraction and clean-up

Total RNA was extracted using Tri reagent (Sigma). For each culture dish, the differentiation medium was removed and the cells lysed by addition of 8ml of Tri reagent. The cell lysates were passed several times through a needle then treated with chloroform and centrifuged. RNA was precipitated from the resulting aqueous phase by addition of isopropanol. 300-400μg total RNA was extracted from each 10cm dish of cells, approximately 100μg of each sample was passed down an RNeasy column (Qiagen), removing contaminating DNA by the use of an ion-exchange matrix, yielding 50-75μg of clean RNA.

2.5.2.2 cDNA synthesis

The Invitrogen Life Technologies SuperScript Choice system was used for cDNA synthesis. Procedures were carried out according to manufacturers protocol with modifications as detailed in the Affymetrix Technical Manual. 20-25μg of clean total RNA was used for cDNA synthesis. First strand synthesis was carried out using the following T7-(dT)24 primer (100pmol per reaction):

5' – GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24 – 3'

This primer contains an oligo dT sequence to hybridise to the polyA tail of the RNA but also incorporates a bacteriophage T7 RNA polymerase promoter into the newly synthesised cDNA. The first strand synthesis reaction with SuperScript reverse transcriptase (30 000units/ml) was carried out at 42°C. Second strand synthesis in the presence of DNA polymerase I, DNA ligase and RNase H was subsequently carried out at 16°C, followed by incubation with T4 DNA polymerase. Clean-up of the cDNA was achieved by phenol/chloroform extraction and ethanol precipitation.

2.5.2.3 In vitro transcription (IVT)

Approximately one-fifth of the total cDNA synthesised for each sample was used in the IVT reaction. The reaction was carried out using the Bioarray High Yield RNA Transcript Labelling Kit (Enzo Life Sciences) according to the manufacturers protocol. cDNA was incubated with T7 RNA polymerase in the presence of biotin-modified ribonucleotides resulting in synthesis of biotin-labelled cRNA from the promoter sequence incorporated during cDNA synthesis. Clean-up of IVT products was carried out with Qiagen RNeasy columns followed by quantitation of RNA using an Agilent Bioanalyser. An adjusted cRNA yield was calculated from the following equation in order to account for carryover of unlabelled total RNA:

$$\text{Adjusted cRNA yield} = RNA_m - (\text{total } RNA_i)(y)$$

RNA_m = amount of cRNA measured after IVT (μg)

Total RNA_i = starting amount of total RNA (μg)

y = fraction of cDNA reaction used in IVT

All samples produced an adjusted cRNA yield of 30-50μg

2.5.2.4 Fragmentation of cRNA

20μg cRNA for each samples was fragmented in 1X fragmentation buffer at a final concentration of 0.5μg/μl by incubation at 94°C for 35 mins. Approximately 15μg was reserved for hybridisation, 1μg was analysed by gel electrophoresis to confirm the approximate size of the fragmentation products (~35-200 bases).

2.5.2.5 Hybridisation

Hybridisation was carried out according to the Affymetrix Expression Analysis Technical Manual. 300µl hybridisation cocktail was made up for each sample containing a final concentration of 0.05µg/µl fragmented cRNA. The cocktail was heated at 99°C for 5 minutes, then 45°C for 5 minutes, followed by centrifugation in a microcentrifuge at maximum speed for 5 minutes. Each MG-U74Av2 probe array was equilibrated at 45°C in 1X hybridisation buffer before removing the buffer and filling the array with 250µl of clarified hybridisation cocktail. The samples were hybridised to the array for 16 hours at 45°C, rotating at 60rpm.

2.5.2.6 Washing and staining

The GeneChip Fluidics Station 400 was used to wash and stain the probe array. The antibody amplification protocol was followed according to the Affymetrix manual.

Following hybridisation, the array was washed first in Buffer A (non-stringent wash buffer) then Buffer B (stringent wash buffer). The array was then stained first with a streptavidin phycoerythrin solution and then with an antibody solution containing goat IgG anti-streptavidin and a biotinylated antibody. Finally the array was stained a second time with streptavidin phycoerythrin. This series of stains and washes initially binds fluorescent labelled streptavidin to the biotinylated cRNA and then amplifies the signal through the antibody step and additional staining. The array was then scanned on the Gene Array Scanner which uses a confocal laser to excite the bound fluorescent molecules and then records the intensities.

Molecular biology grade, RNase-free reagents were used throughout. All pipette manipulations were done using RNase-free plugged tips.

2.5.3 Quality control

During the course of sample preparation, RNA degradation and contamination was checked periodically by gel electrophoresis, spectrophotometry and in an Agilent bioanalyser. At all stages, RNA was of high quality, within Affymetrix recommended parameters.

A series of control parameters were checked to monitor the quality of the data generated. The percentage of probe sets called Present was approximately 50% and was comparable across samples. Average background and raw noise values were within typical range. The 3'/5' ratios for the GAPDH gene were less than 3. Hybridisation controls, E. coli genes spiked into the hybridisation cocktail at a range of concentrations, were all present on the chip. These results collectively verified the quality of the samples and the arrays, and confirmed that all the arrays to be included in the analysis were comparable.

2.5.4 Data analysis

The MG-U74Av2 chip contains 12 488 probe sets representing around 6 000 sequences from the mouse Unigene database and a further 6 000 ESTs. Each transcript is represented by around 16 (often overlapping) probe pairs, the probe sequences are taken from within 500 base pairs of the 3' end. This is because the method of producing the RNA to hybridise to the chip has a 3' bias due to the use of oligo dT primers for the cDNA synthesis. Each probe pair consists of two 25mers, a perfect match and a mismatch, the mismatch has one base different in the centre of the sequence. The difference in the hybridisation intensities between the perfect and the mismatch is considered for each probe pair in the set and used to calculate an overall signal for each of the transcripts represented on the chip, it also allows for calculation of background and cross-hybridisation.

2.5.4.1 Statistical analysis of raw data

After completion of scanning, the raw data files for each chip were analysed by implementation of a number of statistical algorithms, based on standard statistical techniques, in the Affymetrix Microarray Suite 5.0. For each transcript, expression measurements were made based on the relative hybridisation intensities of the perfect and mismatch probe pairs for that specific transcript. A detection call of Present, Marginal or Absent was assigned to each and a Signal calculated to provide a measure of the abundance of the transcript.

In order to compare the transcription profiles of a number of arrays, GeneSpring, a program designed to analyse microarray data for statistically significant changes, was used. Firstly normalisation steps were carried out to enable comparison of the chips. It

is recommended that a per chip normalisation and a per gene normalisation step is carried out. Per chip normalisation considers all transcripts with a Present call (ignoring those called Absent) and divides each chip by the median. For per gene normalisation, each gene is divided by the median value for that gene across all chips.

Following normalisation the data for each was passed through a number of filters, the first to remove genes called absent in all replicates at all time point as this data is uninformative leaving only those genes which are present in at least one time point. These lists were then further filtered by fold change. For each of the three compounds, 109, nocodazole and 401, the ratio between each of the timepoints was calculated and genes which were judged to be unchanged over the entire course of the 24 hour time period were discarded. Anything less than 1.5-fold was considered unchanged. The resulting lists contained probe sets called as Present in at least one time point and altered at least 1.5-fold at some point in the 24 hour experiment.

The next level of filtering was to determine which of the genes altered at least 1.5-fold showed a statistically significant difference between the data at different timepoints. Where arrays had been carried out in triplicate, data was subjected to one-way ANOVA (analysis of variance), a powerful statistical tool capable of comparing data across multiple samples, in this case there are three timepoints for each condition. For data obtained with 401, the single timepoint at 24 hours was done in duplicate and statistical significance was determined by pairwise comparison with time 0 in a Students T-test using Microsoft Excel. In all cases a p-value cut-off of 0.05 was applied.

For 109 and nocodazole, the ANOVA produced a list of genes which showed a 1.5-fold change at one of the timepoints, in order to determine at which time point, post-hoc testing was carried out on the resulting list in Microsoft Excel. Pairwise t-tests were done to determine where the statistically significant difference lay. A p-value of 0.1 was used here as these genes have already been determined to be significant and as ANOVA is a more powerful method there will occasionally be genes which appear to show no significant difference in the post-hoc test at the same level of stringency. In order to pick these genes up, the p-value is increased.

Affymetrix-supplied gene names were updated by cross-referencing with Genbank accession numbers and where more than one name existed the most common or most descriptive was applied. In some cases, a single gene was represented by more than one probe set on the chip, where this occurred the magnitude and direction of the changes were almost identical and each gene is included only once in the final annotated gene lists.

2.5.4.2 Enrichment of functional categories

Using the Netaffx Analysis Centre, each of the three probe set lists (comprising genes regulated by 109, nocodazole or 401) was queried in all fields for each of the category search terms in turn. In addition, the entire 12 488 probe sets on the chip were also searched with the same terms. The percentage of probe sets on the chip in each category was used to calculate the expected frequency in each of the three probe set lists given a random distribution of the genes. Pearson's chi squared test was then applied to determine whether the observed frequency of genes involved in a particular cellular process was significantly different from the expected value. The chi squared value (χ^2) for each category was compared with a table of values of χ^2 for which p=0.05, given 1 degrees of freedom, the null hypothesis that the proportion of genes in a given category arose by chance, was discarded if χ^2 was greater than 3.84.

2.6 MICROSCOPY

2.6.1 Live cell imaging (time-lapse microscopy)

A purpose-built Perspex chamber was constructed to provide a controlled environment in which to maintain mammalian cells for long term time-lapse experiments. Figure 2-1 is a photograph showing the Zeiss Axiovert S100 inverted microscope and surrounding chamber. The microscope was fitted with a H127 motorised stage (Prior Scientific) with movement in X, Y and Z planes (Figure 2-1, B). The culture dish under study was secured in the centre of the stage and covered with a copper coil-heated quartz lid to prevent condensation, humidified CO_2 was supplied via a pipe through a small hole in the heated lid (Figure 2-1, C), a reservoir of water enabled a humid environment to be maintained. Phase contrast, or fluorescence images were captured with an FDI cooled digital camera (Photonic Sciences). Image acquisition and stage movement were

coordinated through Image Pro Plus software, with integrated AFA (automatic fluorescence acquisition) module. Images were captured at each pre-programmed position on the dish every 4-5 minutes, through a 10X objective and merged to form a movie in Image Pro Plus.

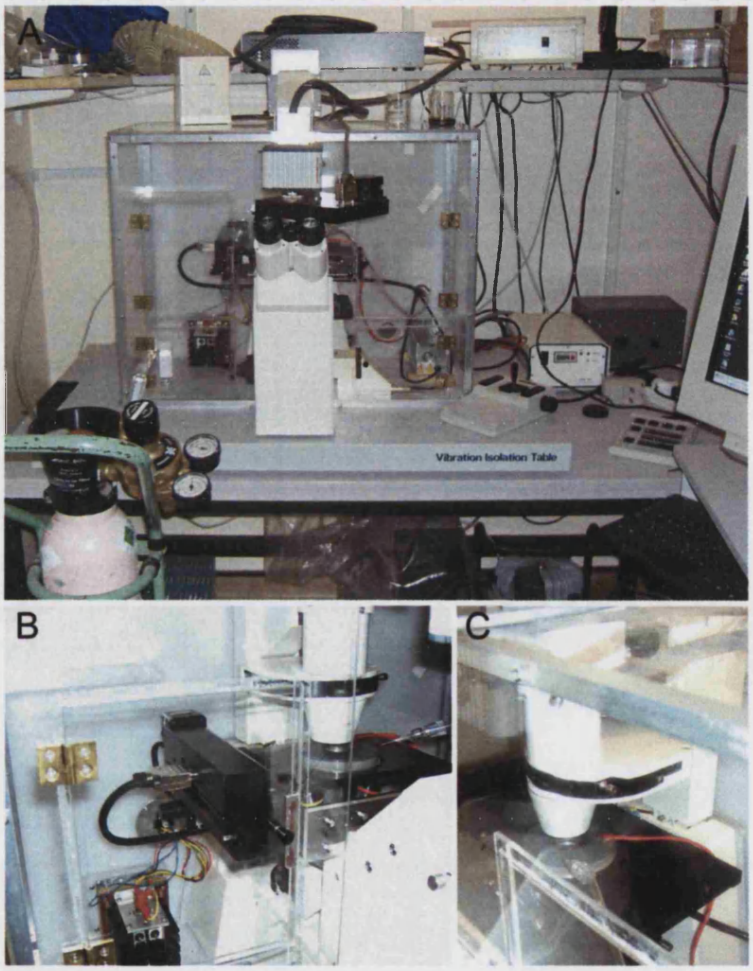


Figure 2-1: Microscope and chamber for time-lapse microscopy. The overall time-lapse arrangement is shown in A, with details from inside the chamber in B and C. During time-lapse microscopy, cells were maintained in a controlled environment by use of a custom-made Perspex chamber, around the microscope stage (A). The cells were supplied with appropriate CO_2 levels from an adjacent cylinder, and mage acquisition, and stage movement was controlled through accompanying computer software. The set-up incorporates a motorised stage (B), which enables different fields on the same dish to be imaged near simultaneously. The cells are placed in the centre of the stage with a surrounding water reservoir and heated lid, through which a tube supplying CO_2 can be passed (C).

2.6.2 Fixed cell imaging

Cells were observed on a Zeiss Axioplan 2 upright microscope and phase contrast or fluorescent images captured using a cooled digital colour camera, Axiocam HRc (Zeiss), controlled through the Axiovision 3.1 software. For fluorescent imaging standard Zeiss filter sets for FITC, rhodamine and hoechst were used. Images of cytoskeletal elements

using a 63X oil objective, were acquired as a z-stack of between 10 and 20 slices through the sample, slices were set at 0.2μm intervals. Further image processing and deconvolution (where appropriate) was carried out using Volocity and Openlab software (both by Improvision).

2.6.3 Quantitation for cellularisation assay

Counts for the cellularisation assay were carried out on a Zeiss Axioskop upright microscope, using a FITC/rhodamine dual band pass filter with a 20X objective.

2.7 BUFFERS

2.7.1 Buffers for immunohistochemistry

1X Extraction buffer
60mM PIPES, pH 6.9
25mM HEPES, pH 6.9
10mM EGTA
1mM $MgCl_2$

2.7.2 Buffers for microarrays

5X Fragmentation buffer
200mM Tris-acetate, pH 8.1
500mM KOAc
150mM MgOAc

The buffer was made up in DEPC-treated water and filtered through a sterile 0.22μm syringe filter.

1X Hybridisation buffer
100mM MES
1M [Na+]
20mM EDTA
0.01% Tween-20

Hybridisation cocktail

300µl was made up for each sample containing:

0.05µg/ml fragmented cRNA

50pM control oligonucleotide B2

1X eukaryotic hybridisation controls

(1.5pM bioB, 5pM bioC, 25pM bioD, 100pM cre)

0.1mg/ml herring sperm DNA

0.5mg/ml acetylated BSA

1X hybridisation buffer

Wash buffer A: NSWB

6X SSPE

0.01% Tween-20

Wash buffer B: SWB

100mM MES

0.1M [Na+]

0.01% Tween-20

Chapter 3

Characterisation of the cellular effects of myoseverin and related compounds

3.1 INTRODUCTION

Myoseverin was isolated from a combinatorial library of several hundred compounds based on morphological changes observed when the compounds were screened on C2C12 myotubes. Constrictions appeared along the length of the myotubes in-between individual nuclei eventually resulting in complete fission and the formation of cells containing one nucleus from a cell that had previously been multinucleate. The microtubule binding activity of myoseverin was subsequently confirmed by an *in vitro* assay but no data revealing the percentage of myotubes affected or the numbers of mononucleates produced was reported. My initial objective was therefore to develop an assay system to quantitate the effect of myoseverin on differentiated myotube cultures. Such a cellular assay can then be used to probe the mechanism of action of myoseverin and determine how its activity may be modified by other molecules or altered by a range of different conditions.

It should be noted that all previous reports on myoseverin describe experiments carried out on mixed cultures. Mononucleate cells were plated at high density and then induced to undergo differentiation *in vitro*. The resulting culture contains a heterogeneous population of mononucleate myoblasts, multinucleate myotubes expressing markers of mature muscle, and cells at various intermediate stages of differentiation. Although these complex cultures are an easy and convenient substrate for assays and for the observation of gross morphological changes as in the original screen, the high density and overlapping nature of the multiple cell types has made it very difficult to visualise the actual process of myoseverin-induced myotube fragmentation. This has left the case for cellularisation open to some criticism and scepticism. An important aim of this work was to demonstrate the action of myoseverin at the level of individual myotubes.

This chapter describes the use of dense mixed cultures to quantitate cellularisation of mammalian myotubes, and of purified cultures to directly visualise the process of cellularisation at a single cell level. I have developed an optimised assay system to quantitate cellularisation of myotubes by myoseverin, and other active compounds, and describe the subsequent use of this assay to investigate some of the characteristics of myoseverin-induced fragmentation of myotubes and the role of protein synthesis, and by implication of gene expression, in the process.

3.2 RESULTS

3.2.1 Design and optimisation of an assay to quantitate cellularisation

3.2.1.1 Designing an assay for cellularisation

Myosin heavy chain (MHC) is a late marker of differentiated muscle and as such is expressed in myotubes after cell cycle withdrawal but is absent from actively dividing myoblasts. MHC is an abundant protein in the myotube and can be detected by immunocytochemistry on fixed cells using a monoclonal antibody that recognises mouse MHC. As the length of assay period was likely to be in the range of 24 hours, MHC, which is expressed in the myotube, should still be detectable in the fragmented myotube following myoseverin treatment. Therefore a count of the number of MHC positive mononucleates with and without treatment would provide a measurable index of fragmentation (Figure 3-1).

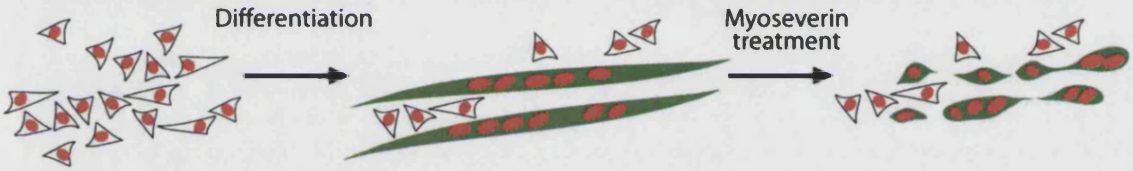


Figure 3-1: Schematic for a cellularisation assay based on the expression of MHC. All nuclei are shown in red, myosin heavy chain (MHC) is represented in green. MHC is absent from the proliferating mononucleates prior to differentiation but is expressed in the multinucleate myotubes formed when the cells are induced to differentiate, and remains detectable in myotube fragments following myoseverin treatment. MHC positive mononucleates can be quantitated by immunohistochemical staining of cells fixed following treatment and used as a measure of cellularisation.

I first determined that myoseverin had no direct effect on the levels of MHC in a population of dividing myoblasts (Figure 3-2, experiments C and D). These counts along with observations of differentiated myotube cultures revealed low background levels of MHC positive mononucleates in all control cultures, these cells appear to have withdrawn from the cell cycle and express MHC but have failed to fuse to form multinucleate myotubes. The variability between control cultures is probably due to differences in cell density and passage number. In order to normalise assay counts across experiments the results are expressed as the percentage of mononucleates staining positive for MHC. As the amount of fusion within a culture can vary, the percentage of MHC positive mononucleates resulting from a particular treatment will often be compared with another condition (e.g. control or another compound) within the same experiment and expressed as a fold change in percentage of MHC positive

mononucleates in order to minimise any artefacts arising from inter-experiment variability.

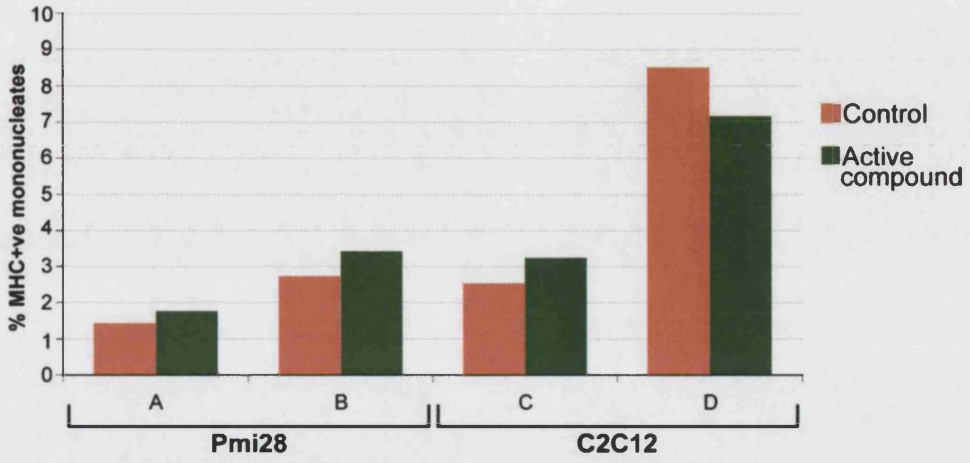


Figure 3-2: The effect of myoseverin on proliferating myoblast cultures. A-D represent four independent experiments carried out on two murine myoblast cell lines. For C2C12 cells the active compound here is myoseverin, while for Pmi28 cells compound 109 was used. Counts of the numbers of MHC positive mononucleates in the presence or absence of active compound are expressed as a percentage of the total number of mononucleates counted.

3.2.1.2 Initial assay development in the C2C12 cell line

I began assay optimisation using the C2C12 mouse myoblast cell line and micromolar concentrations of myoseverin as described in the original work (Rosania et al., 2000). Table 3-1, rows a-g, shows optimisation of assay times. A concentration of myoseverin above 25μM gave noticeably toxic effects. Based on this data, 16 hours was chosen for the assay period, for convenience and the levels of mononucleates produced (it should be noted that the single experiment giving rise to a fold change of 2.14 at a 20hr timepoint, actually showed a 2.35 fold change at 16hrs).

In addition to myoseverin, a number of other purine derivatives and second-generation molecules based around a triazine scaffold were synthesised, by our collaborator Young-Tae Chang (New York University), and made available for assay (Table 3-2). The activity of a panel of compounds compared with that of myoseverin is summarised in Figure 3-3. The graph shows the activity of the compounds relative to 1 (1=activity of 25μM myoseverin). Compound YC67 appears comparable to myoseverin in activity while YC41 and 117 are inactive. Of the purine-based molecules, only YC91 shows an apparent increase in activity over myoseverin, but a degree of cytotoxicity was seen in

the presence of this compound which increased with concentration, explaining the decrease in activity at 10μM. The triazine-based molecules 081 and 109 both show an enhanced ability to fragment myotubes without toxicity.

Table 3-1: Assay optimisation with myoseverin on C2C12 myotube cultures.

	Compound	Conc(μM)	Time(hrs)	% mononucleates MHC+ve		Fold change over control	
				Mean	n	Mean	n
a	None	-	16	4.79	4	-	-
b	None	-	24	2.86	1	-	-
c	MS	20	16	7.17	1	1.22	1
d	MS	25	12	9.65	1	-	-
e	MS	25	16	8.37	7	1.95	7
f	MS	25	20	6.13	1	2.14	1
g	MS	25	24	4.64	1	1.62	1
h	YC41	25	16	4.79	4	-	-
i	YC41	25	24	2.37	1	0.83	1
j	109	15	16	10.08	1	2.47	1
k	109	20	16	8.54	1	1.45	1

Differentiated cultures were treated with myoseverin (MS) over a range of concentrations and time points, and the percentage of MHC positive mononucleates counted. Maximal response without toxicity appears to be achieved with a 25μM concentration over 16 hours. A number of other compounds were also tested, the results for two of these, YC41, an inactive myoseverin derivative, and 109, a triazine based compound with enhanced cellularisation activity are included in this table.

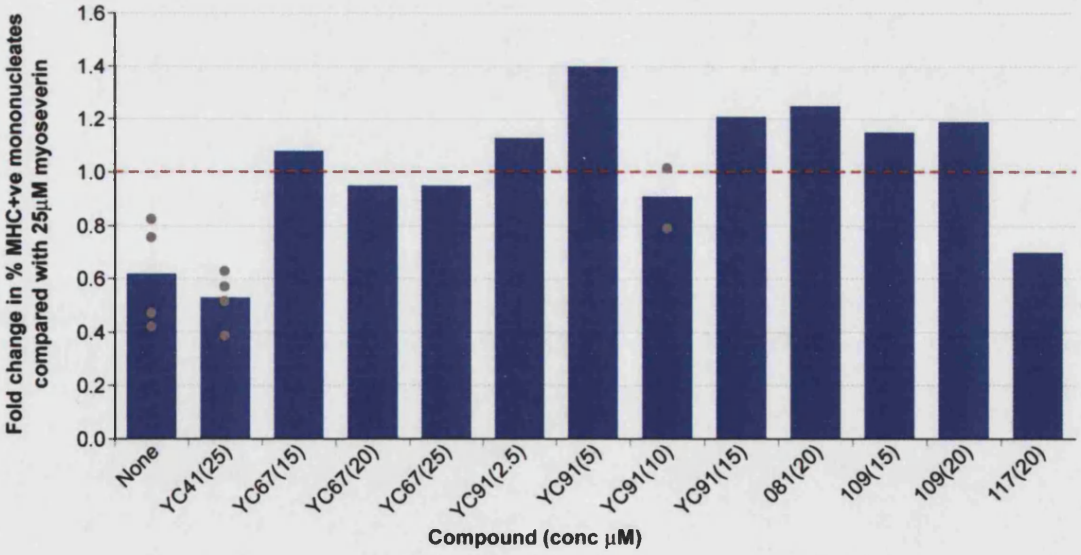
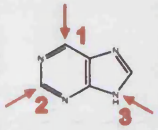
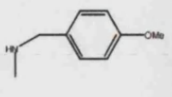
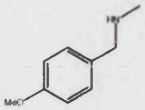
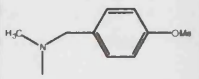
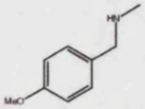
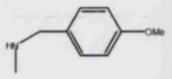
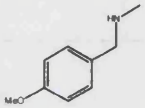
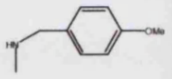
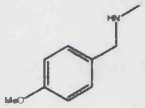
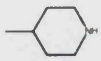
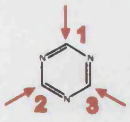
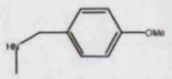
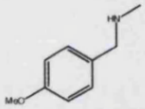
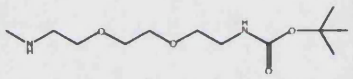
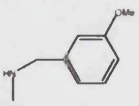
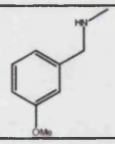
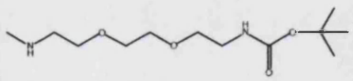
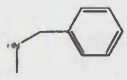
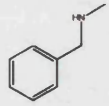
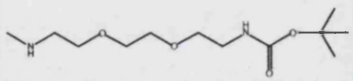
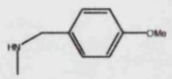
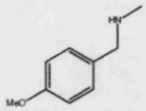
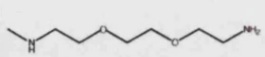
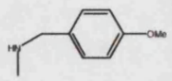
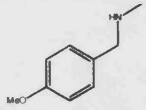
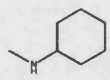
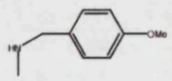
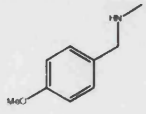
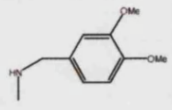
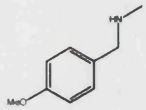
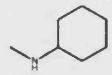


Figure 3-3: Comparison of the activity of a panel of compounds on C2C12 myotube cultures with that of myoseverin. A range of compounds was tested on C2C12 cultures for cellularisation activity in parallel with myoseverin. The results are expressed here as the fold change in percentage of MHC positive mononucleates over myoseverin. All activities are relative to a value of 1.0; a fold change above 1.0 indicates an increase in activity over that of myoseverin. Where replicates were carried out individual experiments are shown as points and the bar represents the average. The remainder were assayed as single determinants but note that multiple concentrations were used, reinforcing the result.

Table 3-2: Chemical structures of purine and triazine based compounds tested in the cellularisation assay.

Base	Compound	Substituent groups		
		1	2	3
 purine	Myoseverin			
	YC41			
	YC67			
	YC91			
 triazine	109			
	401			
	402			
	117			
	081			
	TA			
	TC			

A range of myoseverin derivatives and second-generation molecules based on a triazine ring were synthesised by Young-Tae Chang (New York University) and made available for testing in my assay.

Compound YC41 differs in structure from myoseverin by only a single methyl group (Table 3-2) but shows no increase in MHC positive mononucleates in the fragmentation assay, and no visible morphological changes, thus providing an excellent control compound. Statistical analysis of the percentage of MHC positive mononucleates in the absence of any compound, compared with the percentage arising from treatment with YC41 showed no significant difference between the two conditions ($p=0.05$) confirming that YC41 is completely inactive on myotubes. With both YC41 and in the absence of any compound, there is a slight decrease in the percentage of MHC positive mononucleates between the 16- and 24-hour time points. This is probably due to the continuing fusion of mononucleates in differentiation medium over time. Although the percentage of MHC positive mononucleates with 25µM myoseverin compared with either the control compound YC41 or with no compound at all was judged to be statistically significant in a t-test ($p=0.02$), the average fold increase is less than two-fold (Table 3-1, row e) and even with compound 109, the most active of the newly synthesised molecules, a maximal fold increase of only 2.47 was observed. Trying to develop a system that would give a greater range to work with, led me to consider using an alternative cell line. Intuitively it would seem more conducive to fragmentation if nuclei were arranged in a single row along the length of a long narrow myotube. The C2C12 cell line often fuses to give very large, wide myotubes containing clusters of nuclei at the centre. In contrast, the Pmi28 cell line forms myotubes with a morphology that would seem much more favourable to fragmentation with myoseverin (Figure 3-4).

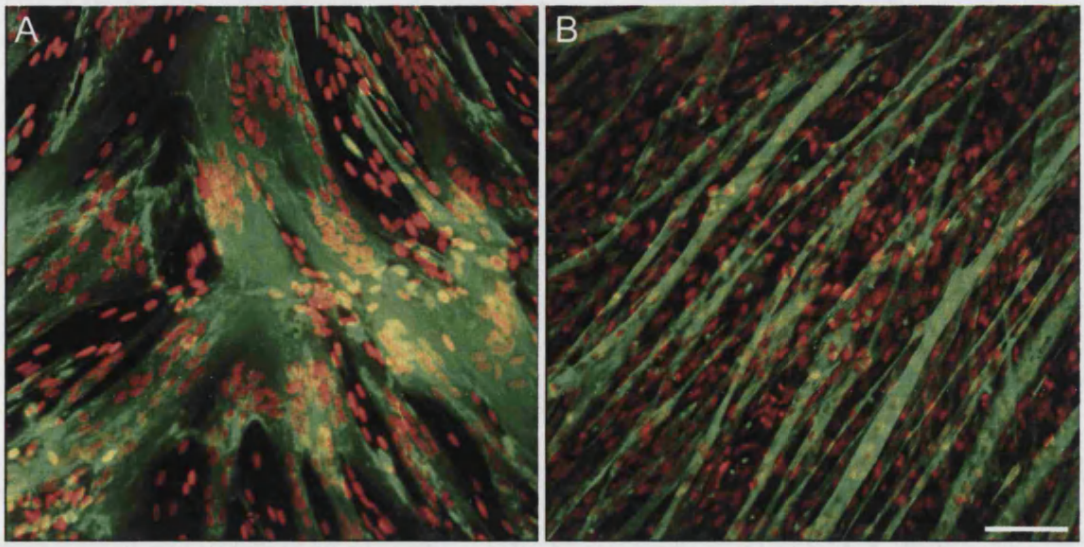


Figure 3-4: Comparison of myotube morphology in the C2C12 and Pmi28 cell lines. Immunofluorescence on fixed cells stained with an antibody to MHC (green) and with the nuclear stain propidium iodide (red), shows large multinucleate C2C12 myotubes with clusters of nuclei (A), while Pmi28 cells form long parallel myotubes containing a single column of nuclei (B). Scale bar = 100µm

3.2.1.3 Assay optimisation with Pmi28 cells

The mouse myoblast cells Pmi28 are derived from a primary culture of hind limb muscle from a 7-day-old male Balb/c mouse (Irintchev et al., 1997). The cells were initially expanded and enriched and shown to successfully contribute to muscle fibres and satellite cell populations following intramuscular or subcutaneous implantation into immunologically compatible hosts (Irintchev et al., 1997; Irintchev et al., 1998; Link et al., 2001). Pmi28 cells have subsequently been propagated for over 40 passages with no loss of myogenic or growth properties (Irintchev et al., 1997) and have also been used for *in vitro* studies as a model for skeletal muscle (Kaufmann et al., 1999; Link et al., 2001; Storz et al., 1998).

Both the C2C12 and Pmi28 cell lines have very similar fusogenic potential with around one-third of the nuclei present in multinucleate myotubes after 3-4 days in optimal differentiation conditions as shown in Table 3-3.

Table 3-3: Distribution of nuclei in differentiated myogenic cultures.

	% of total nuclei present in each cell type		
Cell line	MHC-ve mononucleate	MHC+ve mononucleate	Myotube
C2C12	61.73	2.97	35.30
Pmi28	63.57	1.54	34.89

The results shown here represent the mean of two independent experiments for C2C12 cells and six independent experiments for Pmi28 cells. Differentiated cultures were fixed and stained for MHC; nuclei were counterstained with propidium iodide. In each experiment, between 1000 and 4000 nuclei from randomly chosen fields were counted and scored as either mononucleate or within a myotube, and as MHC positive or negative. All myotubes were MHC positive. Fusogenicity of the two cell lines is almost identical as evidenced by the percentage of nuclei present within myotubes, but C2C12 cultures contain almost twice the levels of MHC positive mononucleates.

Significantly, however, the background level of MHC positive mononucleates in Pmi28 cultures is almost half that for the C2C12 cell line (Table 3-1, rows a-b and Table 3-4, rows a-b). As compound 109 had previously been shown to have enhanced fragmentation ability, further assay optimisation was carried out using this compound and the alternative Pmi28 cell line (Table 3-4). A concentration of 15μM over an assay period of 24hrs was chosen as optimal. Although a longer assay period resulted in a larger number of MHC positive mononucleates, this was accompanied by cytotoxicity at 40- and 48-hour time points. A series of myoseverin and triazine based derivatives were tested on Pmi28 cultures as for C2C12, their activities are summarised in Figure 3-5.

Table 3-4: Assay optimisation with compound 109 on Pmi28 myotube cultures.

	Compound	Conc(μM)	Time(hrs)	% mononucleates MHC+ve		Fold change over control	
				Mean	n	Mean	n
a	None		16	2.47	6	-	-
b	None		24	1.93	11	-	-
c	109	10	8	8.37	1	2.46	1
d	109	10	12	10.33	1	3.04	1
e	109	10	16	12.36	2	3.49	2
f	109	15	12	4.78	1	1.94	1
g	109	15	16	9.25	9	4.90	5
h	109	15	20	8.82	2	3.32	1
i	109	15	24	11.03	12	5.73	10
j	109	15	40	14.95	1	-	0
k	109	15	48	22.85	1	11.09	1
l	109	20	16	11.78	1	3.18	1
m	401	15	24	1.69	2	0.78	2
n	402	15	24	1.28	2	0.60	2
o	MS	25	16	9.26	1	2.50	1

Differentiated Pmi28 cultures were treated with 109 at a range of concentrations and times. 15μM 109 over 24 hours gives rise to an increased percentage of MHC positive mononucleates without the toxicity seen at higher concentrations or extended time periods. Results initially obtained with C2C12 cultures, and confirmed here, show that 109 is more active in the cellularisation assay than myoseverin. 401 and 402 are both structurally similar to 109 but are inactive on myotubes.

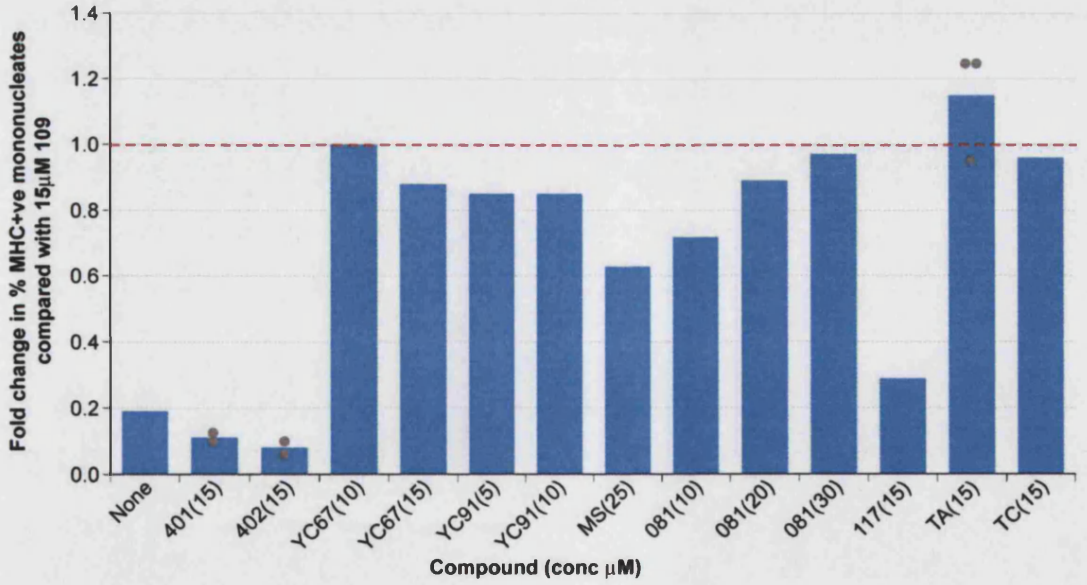


Figure 3-5: Comparison of the activity of a panel of compounds on Pmi28 myotube cultures with that of 109. A range of compounds was tested on Pmi28 cultures for cellularisation activity. The percentage of MHC positive mononucleates for each was compared directly with results obtained in parallel with 109 and expressed as a fold change. All activities are relative to 1.0; a fold change above 1.0 indicates an increase in activity over that of 109. Where replicates were carried out individual experiments are shown as points and the bar represents the average. The remainder were assayed as single determinants but note that many of the compounds were tested at multiple concentrations, or in multiple cell lines (see Figure 3-3), thus reinforcing the result (for None, n=10, SD=0.06, not shown in figure).

Compound YC67 gave results comparable with those of 109, while 081 induced cellularisation but with reduced potency. Compound TA (a newly synthesised compound not available earlier for testing on C2C12 cells) was the only compound that appeared to show a slight increase in activity over 109. Compounds 401 and 402 were inactive and showed no significant difference from no compound at all in a t-test ($p=0.05$). Compound 401 provides an excellent control molecule for experiments with 109 as its molecular weight is identical to that of 109, the two molecules differ only in the positioning of methoxy groups on a benzene side group (Table 3-2, substituent groups 1 and 2). Figure 3-6 shows Pmi28 myotubes treated with 401 or 109 and demonstrates that 401 brings about no observable changes in the culture. Given the availability of such an appropriate control, and the relatively small increase in activity afforded by TA, compound 109 (at a concentration of 15µM and over a 24hr time period) will be used for all my subsequent investigations into the mechanism of cellularisation.

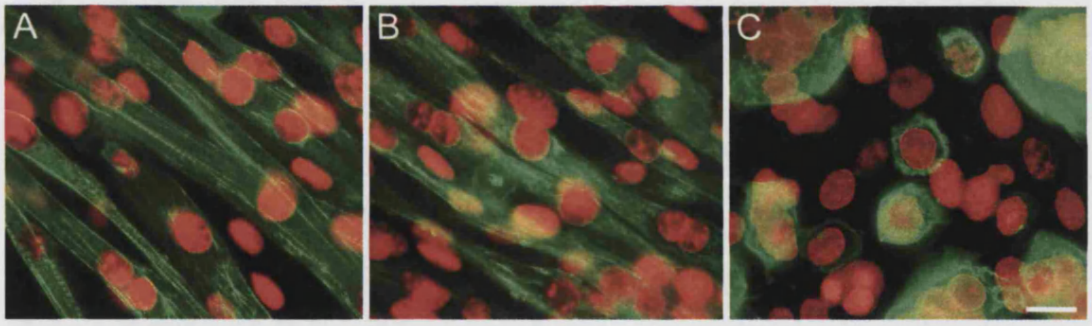


Figure 3-6: Immunofluorescence of Pmi28 myotube cultures treated with compounds 109 and 401. Cells were untreated (A), or treated with 15µM 401 (B) or 15µM 109 (C) for 24 hours, then fixed and stained for MHC (green) and with the nuclear stain propidium iodide (red). Compound 401 is completely inactive and has no effect of myotube morphology while 109 brings about fragmentation and cellularisation of the myotubes. Scale bar = 20µm.

Figure 3-7 shows the comparison between the original C2C12 cell system with myoseverin as the active compound and the Pmi28 cells with 109. 109 is more active than myoseverin on differentiated cultures of both cell lines tested and both compounds give rise to a greater degree of cellularisation in Pmi28 cultures.

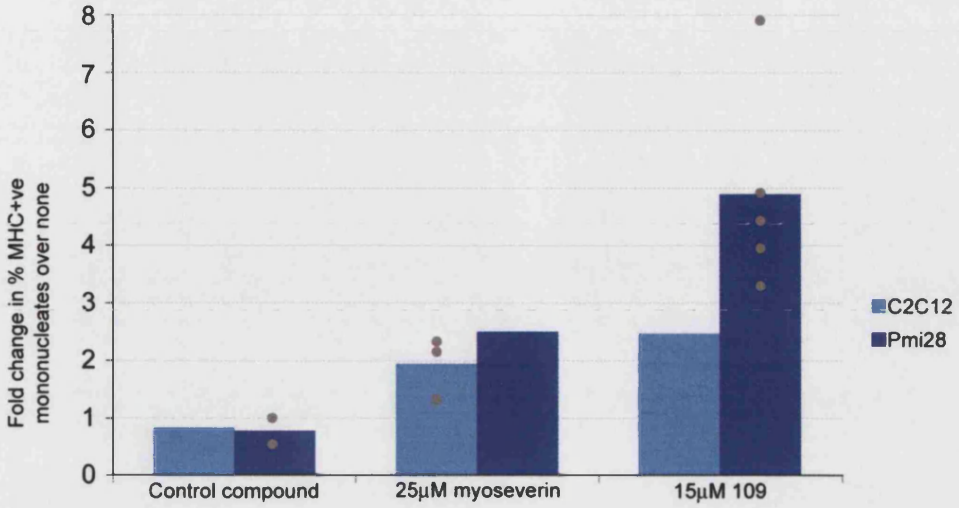


Figure 3-7: Summary of assay optimisation. The percentage of MHC positive mononucleates is expressed here as the fold change in treated over untreated cultures. The control compound is 25µM YC41 for C2C12 cells and 15µM 401 for Pmi28 cells, the assay period is 16 hours. The Pmi28 cell line gives enhanced cellularisation with both myoseverin and 109 and a direct comparison of the two active compounds shows 109 to be more potent. Replicates are shown as individual points while the bars represent the mean (see Table 3-1 and Table 3-2 for details of experiments).

3.2.2 Reversibility of cellularisation

Following fragmentation with 15µM 109 for 24 hours, differentiated cultures were washed gently with D-PBS and incubated in fresh differentiation medium. After only 24 hours, the effects of 109 on cell morphology had been almost completely reversed (Figure 3-8). The images shown in Figure 3-8 are stills from a time-lapse experiment which is illustrated in video1A and video1B in Supporting Data.

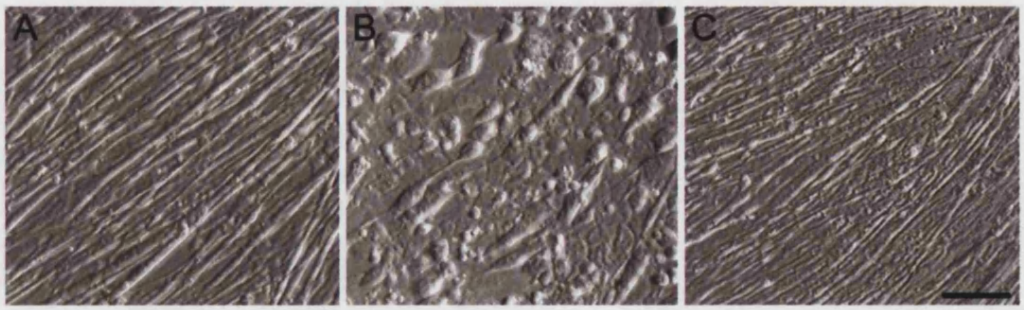


Figure 3-8: Reversibility of 109-induced cellularisation. Phase contrast images from a time-lapse sequence of a differentiated Pmi28 culture before treatment (A), after 16 hours treatment with 15µM 109, immediately prior to washing out the compound (B), and 24 hours in differentiation medium following removal of 109 (C). The same field was imaged throughout. Scale bar = 100µm.

Prior to treatment with the compound, the culture consisted of a mixture of densely packed mononucleates and long narrow myotubes, often aligned with one another and

containing multiple nuclei running the length of the cell (Figure 3-8 A). 109 treatment brought about dramatic changes, myotubes pulled apart to produce smaller multinucleate fragments or mononucleates and contracted into rounded myotubes with nuclei clumped together in the centre (Figure 3-8 B and video1A). On removal of the compound these rounded up myotubes gradually straightened out and most returned to their previous shape, aligning as elongated cells with nuclei rearranging as before along the long axis of the cell (Figure 3-8 C and video1B). The cellularisation assay revealed that the percentage of MHC positive mononucleates rose after 24 hour treatment with 109 as expected but then decreased almost to background levels within 24 hours of washing out the compound (Figure 3-9). This suggests that the population of mononucleates arising from 109-induced cellularisation are competent to fuse once again to form myotubes under differentiation conditions.

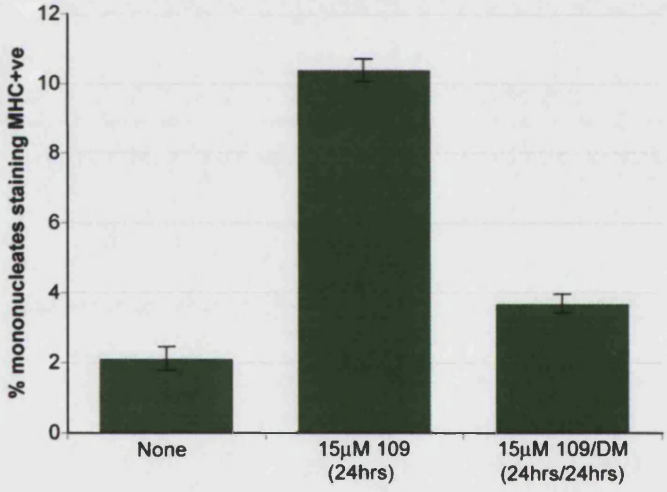


Figure 3-9: Quantitation of cellularisation in Pmi28 cultures following reversal of 109. The increased percentage of MHC positive mononucleates brought about by 109-induced cellularisation decreases when the culture is incubated in fresh differentiation medium (DM) for 24 hours following a 24 hour treatment with 109. The results represent an average of four independent experiments, standard deviation is shown.

3.2.3 Dependence of the action of 109 on *de novo* protein synthesis

I used the protein synthesis inhibitors anisomycin and cycloheximide to assess the dependence of the action of 109 on *de novo* protein synthesis. The results of these experiments will have clear implications for the interpretation of gene expression data and the emphasis that can be placed on such data. A concentration of inhibitor achieving the maximal level of protein synthesis inhibition was found by incubating Pmi28 myotube cultures in the presence of tritiated leucine for 6 hours. Treated dishes were

then processed for autoradiography and the level of radioactive amino acid incorporation was used as a measure of protein synthesis by quantitating the number of grains in the emulsion above the myotubes. Leucine was chosen as it is involved in few metabolic pathways and 80% of the leucine in cells is incorporated into proteins rather than into metabolites (Lipton and Raley-Susman, 1999). Tritium is a low beta emitter and in conjunction with K5 emulsion gives a highly sensitive system (Rogers, 1979). Anisomycin proved to be the more potent inhibitor of the two in agreement with published reports (Cano et al., 1994; Lewis and Mathews, 1980) and at 10µM, a concentration that showed no cytotoxicity, inhibited protein synthesis to 93% (Figure 3-10).

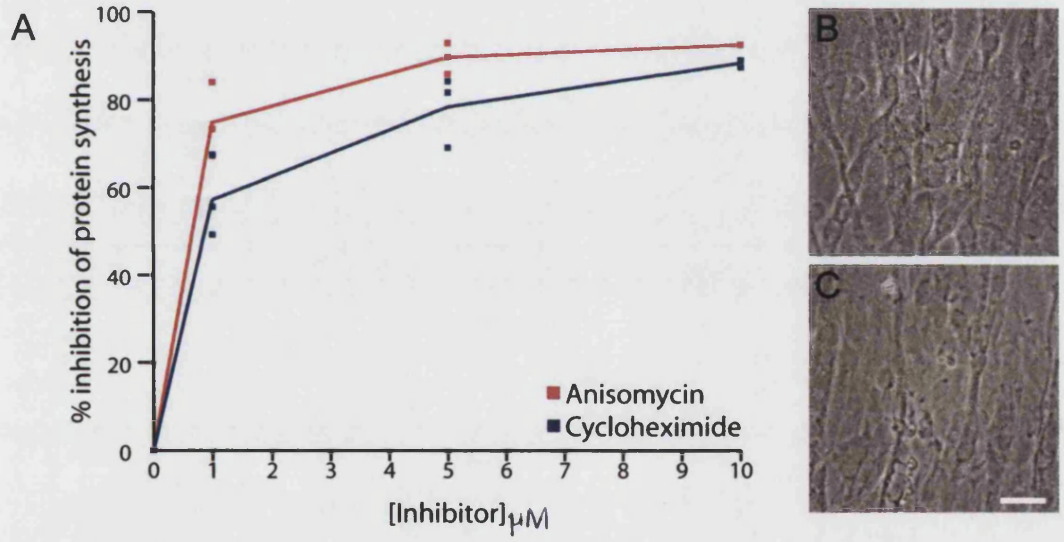


Figure 3-10: Inhibition of protein synthesis in Pmi28 myotube cultures. Inhibition of protein synthesis was quantitated by measuring incorporation of tritiated leucine into myotubes in the presence of increasing concentrations of anisomycin or cycloheximide for six hours (A). Phase contrast images of untreated (B) and 10µm anisomycin treated (C) cultures following autoradiography for detection of tritiated leucine. The white specks are silver grains in the emulsion above the cells and were quantitated to provide a measure of protein synthesis as described in Materials and Methods. Each concentration was measured in triplicate, individual points represent three independent experiments while the line passes through the mean. Scale bar = 20µm.

Anisomycin and cycloheximide both block translational elongation by binding to the 80S ribosome but act at different steps in the process. Cycloheximide inhibits the translocation reaction of the 60S ribosomal subunit while anisomycin specifically inhibits binding of the 3' of the aminoacyl-tRNA to the active site of the peptidyltransferase (Meskauskas and Dinman, 2001). It is now known that these drugs also affect other processes in the cell such as apoptosis and kinase signalling (Cano et al., 1994; Shifrin

and Anderson, 1999; Torocsik and Szeberenyi, 2000). Anisomycin and cycloheximide are often used in the same experiment to control for non-specific effects (Martin et al., 1988; Torocsik and Szeberenyi, 2000), as they act by different mechanisms both inhibitors are tested to ensure that effects seen are due to protein synthesis inhibition.

Assay results revealed a decrease in fragmentation by 109 in the presence of anisomycin and cycloheximide. Table 3-5 shows that the inhibitors alone are inactive in the assay while in the presence of 109 they clearly attenuate the action of 109 but do not abolish it completely.

Table 3-5: The effect of protein synthesis inhibition on the percentage of MHC positive mononucleates resulting from 109 treatment.

Treatment	% MHC+ve monos	
	Mean	n
None	1.69	3
10μM anisomycin	1.64	1
10μM cycloheximide	1.83	1
15μM 109	8.93	4
15μM 109 + 1μM anisomycin	4.37	2
15μM 109 + 5μM anisomycin	4.40	2
15μM 109 + 10μM anisomycin	5.14	1
15μM 109 + 5μM cycloheximide	4.70	2
15μM 109 + 10μM cycloheximide	5.78	1

Cells were treated with 109 in the presence of anisomycin and cycloheximide and the degree of cellularisation assayed.

By subtracting the background levels of MHC positive mononucleates to give an actual measure of mononucleates arising only from fragmentation, and then comparing the levels achieved by 109 in the presence and absence of inhibitor, it is possible to express the results in Table 3-5 in terms of the inhibition of 109 activity when protein synthesis is severely disrupted (Figure 3-11). Some fragmentation of myotubes is still able to occur even with 93% inhibition of protein synthesis but there is no doubt from these results that *de novo* protein synthesis greatly facilitates the action of 109. Observations of cell cultures fixed and stained for MHC following 109 treatment in the presence of anisomycin or cycloheximide reveal some contraction and rounding up of myotubes and cells as with 109 alone, while a small proportion of myotubes seem to be in the process of fragmentation but remain attached by narrow cytoplasmic bridges (Figure 3-12). The inhibition of fragmentation by anisomycin and cycloheximide correlates with their

activity as inhibitors of protein synthesis; anisomycin is more effective at blocking fragmentation and for both compounds their greatest effect is at higher concentrations.

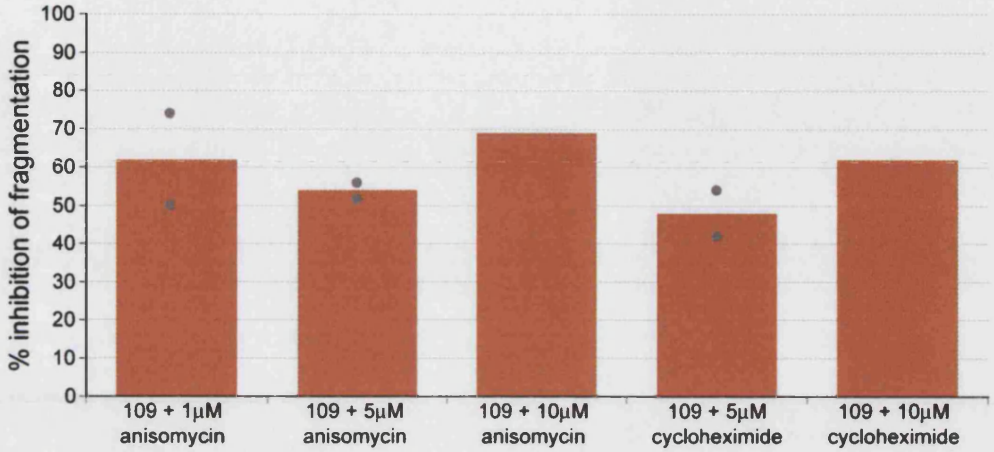


Figure 3-11: Inhibition of 109-induced cellularisation by anisomycin and cycloheximide. The assay counts for treatment with 109 in the presence of cycloheximide and anisomycin are represented here in terms of percentage inhibition of cellularisation occurring with 109 alone. Duplicate experiments are shown as individual points while the bar represents the mean.

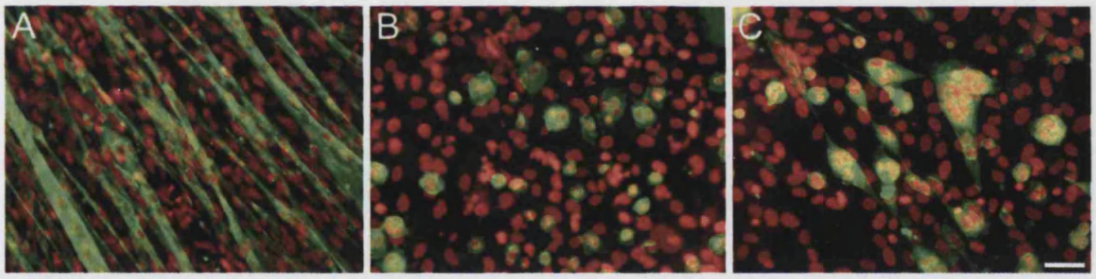


Figure 3-12: Treatment of Pmi28 myotubes with 109 in the presence and absence of the protein synthesis inhibitor anisomycin. Immunohistochemical staining of differentiated cultures treated with no compound (A), 15μM 109 (B) or 15μM 109 in the presence of 10μm anisomycin (C). MHC staining is in green and nuclear staining with propidium iodide in red. Note that in the presence of anisomycin some of the cells remain joined by narrow cytoplasmic bridges. Scale bar = 50μm.

3.2.4 Demonstration of cellularisation at the single cell level

As mentioned previously, all experiments described so far (including the original work by Rosania et al) were carried out on bulk cultures containing a mixture of MHC negative mononucleates, multinucleate myotubes and a low background level of MHC positive mononucleates, the relative proportions of these cell populations are shown in Table 3-4 as described earlier. The high density and complexity of a culture containing cells of differing size and morphology has made it very difficult to visualise the process of myotube fragmentation and there has, as yet, been no demonstration of cellularisation

with either myoseverin or 109 at the level of a single cell. It was therefore important to show in this work the action of 109 on individual myotubes, thus eliminating any doubts that the increase of MHC positive mononucleates quantitated in the assay was due to a genuine cellularisation event.

Time-lapse microscopy was employed to enable visualisation of the action of 109 over a 24-hour period. As it can be difficult to distinguish the nuclei within a myotube using phase contrast imaging alone, Pmi28 cells were transfected with a pseudotyped retroviral vector carrying an eGFP reporter construct with a nuclear localisation signal to target the eGFP protein to the nucleus (Yee et al., 1994). Mononucleates were transfected with ~50% efficiency; the transfected cells proliferated, fused to produce myotubes, and showed no detectable alterations in growth or differentiation characteristics (Figure 3-13, A and B). On fusion it was estimated that greater than 50% of the nuclei within myotubes were positive for GFP. No cytoplasmic GFP was detected by fluorescence microscopy.

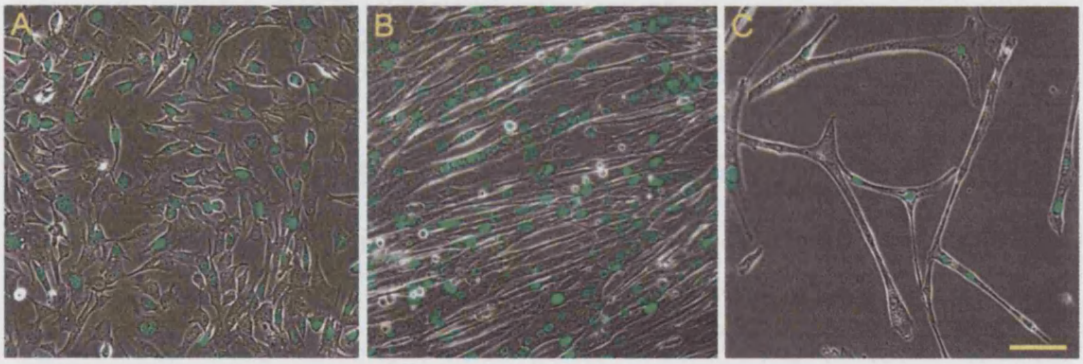


Figure 3-13: Pmi28 cells expressing a nuclear eGFP protein. Phase contrast and fluorescence live cell imaging of Pmi28 cells transfected with a pseudotyped retroviral vector encoding a nuclear localised eGFP. Proliferating myoblasts were transfected (A), induced to form myotubes in culture (B), and then purified (C) to enable clearer visualisation of individual myotubes. Scale bar = 100µm.

In order to reduce the complexity of the culture, cells were cultured for three days under normal differentiation conditions and then purified by passing the cells, in suspension, through a series of filters, to reduce cell density and enrich for myotubes (Figure 3-13, C). The cells were then placed under the microscope in a chamber with controlled environmental conditions at 37°C and 5% CO_2. Image capture was begun immediately following addition of 15µM 109 to the dish, with near simultaneous phase contrast and eGFP fluorescent images of the same field of myotubes taken every 4 or 5 minutes for 24

hours. A composite movie sequence of the two channels was later generated using Image Pro Plus software. Figure 3-14 and video2 (see Supporting Data) are the results of one such time-lapse recording. This experiment was repeated multiple times with both eGFP and untransfected cells to confirm that morphological changes were not a result of exposure of cells to u.v. radiation.

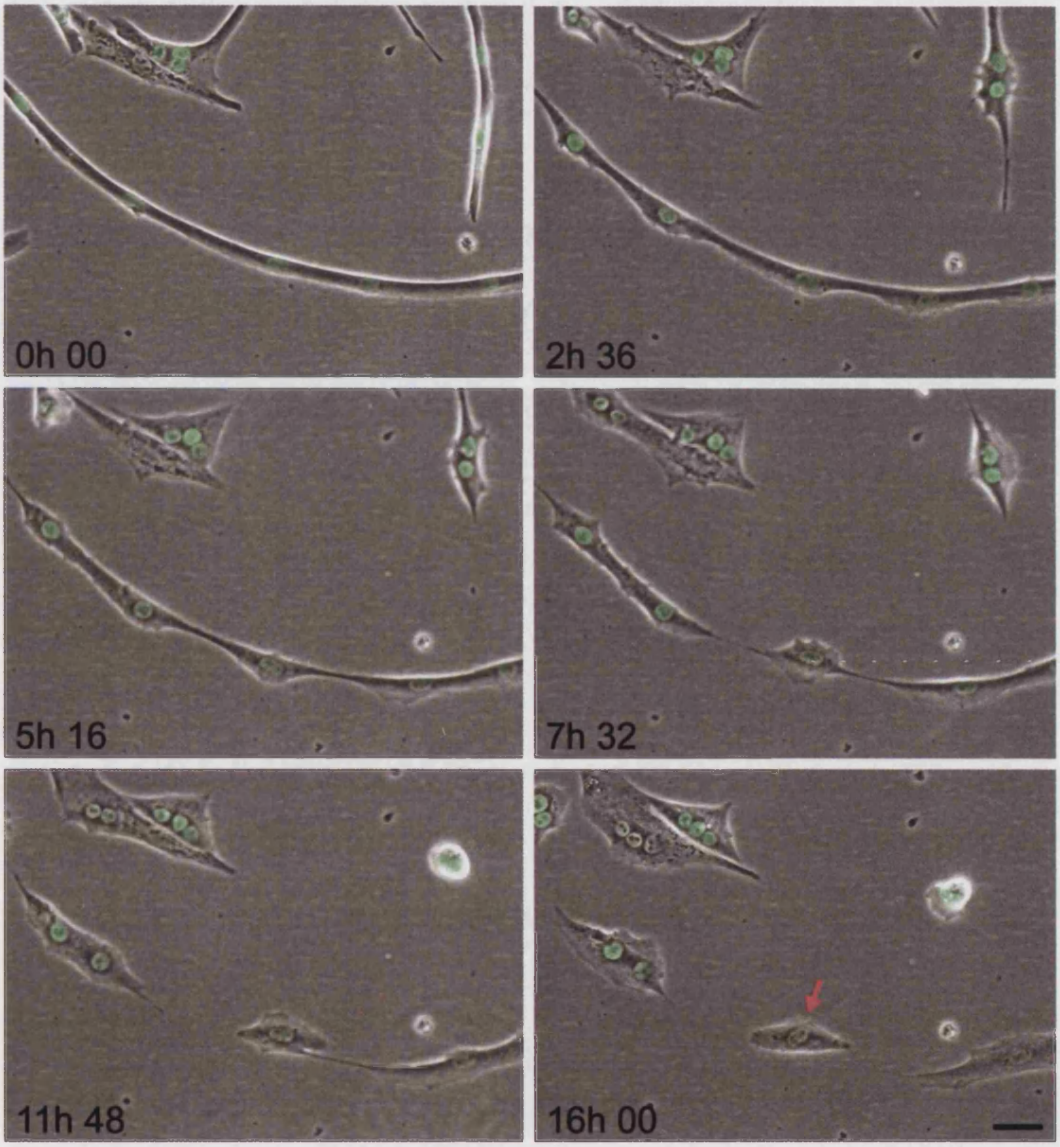


Figure 3-14: Demonstration of cellularisation of Pmi28 myotubes by 109 at a single cell level. Pmi28 myotubes expressing nuclear eGFP were purified and individual myotubes were then imaged by dual channel time-lapse microscopy over a 16 hour period of treatment with 15μM 109. Selected frames from a time-lapse sequence are shown here to illustrate the progression of cellularisation. The arrow indicates the resulting mononucleate cell. Scale bar = 50μm.

Cellularisation can clearly be observed in both video2 and the stills in Figure 3-14, an initial response to 109 is seen almost immediately, there is nuclear movement as the two

ends of the myotube contract away from each other, leaving a single nucleus caught between two regions of cytoplasmic constriction. After 7h 30, one of these narrow cytoplasmic bridges breaks completely severing the emerging mononucleates from one half of the myotube, six and a half hours later the bridge connecting the single nucleus to the rest of the myotube also breaks completely leaving a single mononucleate cell.

Some myotubes merely contracted into rounded, more globular looking myotubes, others broke up into multinucleate fragments and/or gave rise to one or more mononucleates. Mononucleates arose by fragmentation at all levels along the length of a myotube with some forming from the ends and others, such as the cell in Figure 3-14, from the centre, giving the process a fairly stochastic appearance. Further data on the frequencies of these fragmentation events is included in Chapter 6 of this thesis.

3.3 DISCUSSION

The focus of the work in this chapter was to visualise the actual process of cellularisation of mammalian myotubes by myoseverin at a single cell level and to develop a method for quantitation of these events in an optimised cell culture system. The original work published on myoseverin lacked both a clear demonstration of cellularisation and any data to suggest the extent of myotube fragmentation. Both were clearly issues that needed to be addressed before more extensive work into the mechanism of action of this small molecule could reasonably be carried out.

The basis of the assay was to be the expression of MHC, a protein present only in post-mitotic myotubes and retained in mononucleate cells produced by myoseverin-induced fragmentation of the myotubes, thus allowing these newly formed mononucleates to be distinguished from myoblasts already present in the culture. As the differentiated cultures used in the assay contained myoblasts as well as myotubes it was essential to check the effect of myoseverin on MHC expression in myoblasts alone, to confirm that any increase in MHC positive mononucleates was due solely to cellularisation of myotubes. Experiments done in duplicate on C2C12 and Pmi28 myoblasts show a slight increase in the percentage of MHC positive mononucleates for Pmi28 cells. With C2C12 cells one experiment showed a slight increase and one a slight decrease. These small

fold increases are not significant in a t-test (p=0.05) and cannot account for the much larger increases seen in the presence of myotubes. The slight increase in percentage of MHC positive mononucleates in the presence of 109 may instead result from an increase in the number of MHC negative cells in the control culture as the addition of 109 prevents cells from dividing (as do several other known microtubule-binding molecules). In the differentiated cultures used in the assay, cells are at 100% confluence to encourage fusion so all cell division is limited by contact inhibition. It was not feasible to carry out these control experiments on confluent mononucleates as it would be impossible to prevent fusion. I conclude that these small differences between treated and untreated myoblasts are likely to be due to experimental variability and are not significant enough to challenge the validity of the assay.

Initial work on assay development was carried out with C2C12 cells but examination of an alternative mouse myogenic cell line revealed that Pmi28 cells were a potentially more sensitive and efficient system for the assay. The C2C12 cell line is widely used as a model for myogenic differentiation *in vitro* and was used in the screen from which myoseverin was identified. However, a cell line derived almost three decades ago (Yaffe and Saxel, 1977) is open to criticism over the effects of long-term culture on the properties of the cells. The Pmi28 cells were derived far more recently than the C2C12 line (Irintchev et al., 1997), and have been used preferentially in some studies because of the belief that these cells more closely represent the *in vivo* system (Kaufmann et al., 1999; Storz et al., 1998). In the context of my study, the Pmi28 cells certainly appear to have a more physiologically relevant morphology. The myotubes tend to be long and straight with nuclei running along the length, and often align together in parallel much as myofibres in an intact muscle. The cells also overlap and overlay one another giving a three-dimensional appearance when compared to the more artificial monolayer seen with the C2C12 cell line in my hands. These properties of Pmi28 myotubes appear to make them a better substrate for fragmentation by myoseverin, and of particular relevance in the context of this assay was the observation that the level of MHC positive mononucleates in control cultures was less for Pmi28 cells than for C2C12 cells. The problems of conducting the assay within a C2C12 background became apparent when I began to look at the effects of protein synthesis inhibitors on the action of myoseverin. As the data shown for Pmi28 cells indicates, the inhibition of protein synthesis led to

intermediate levels of MHC positive mononucleates. The C2C12 cells routinely gave only 2-fold changes between myoseverin and the control compound YC41, which, although judged significant by a t-test, gave a very narrow range to work in and made it difficult to interpret the significance of treatments (such as protein synthesis inhibition) which generated fold changes of less than 2-fold in an assay which, because of its cellular basis, is subject to an inherent degree of variability.

In addition to discovering a more optimal cell line I also had available to me a number of alternative purine and triazine based molecules, designed from structure:activity relationship studies on myoseverin. Although myoseverin was originally isolated from a library designed around a purine molecule (Chang et al., 1999), the synthesis of a second series of molecules with the triazine ring at its centre but possessing the same activity as myoseverin, shows that for this cellularisation activity the purine backbone is not important. The triazine molecule has three-fold symmetry and is therefore much easier to work with chemically. The two methoxy-benzene substituents, on the other hand, appear to be absolutely necessary for activity as any modifications to these groups result in a severe decrease in activity (Perez et al., 2002; Rosania et al., 2000). The triazine compound 109 was shown to have the same activity as myoseverin but was more potent in my assay and so will be used in almost all subsequent studies into the mechanism of cellularisation of mammalian myotubes brought about by these small microtubule-binding molecules.

Figure 3-7 summarises the significant improvements of my optimised cell culture system for studying cellularisation events in mammalian myotubes compared with the system in the original report. The increase in range afforded by the use of the Pmi28 cells in conjunction with an optimised compound allowed me to identify more subtle changes to the activity of 109 despite some cell variability.

Myoseverin and 109 are known to disrupt the microtubule cytoskeleton, a network that is essential for many cellular processes, and its prolonged disruption has implications for viability, cell cycle progression and differentiation of all cell types in the culture. To enable future characterisation of this newly formed population of mononucleates I hoped

to be able to wash out 109 and establish the reversible nature of its action so that these, and other, aspects of cell behaviour could be investigated.

The use of time-lapse microscopy clearly revealed striking changes in cellular morphology following the removal of 109 after a period of treatment. The observations were indicative of a reversal of the effects of 109, a suggestion further supported by assay data. The MHC molecule is very abundant in the cell and would require several days to completely turnover within the cell, this makes it unlikely that the decrease in MHC positive cells, after washing out 109 and incubating the cells in differentiation medium for a further 24 hours, is due to loss of MHC from those cells produced by fragmentation of myotubes. I propose that the mononucleates produced by cellularisation of myotubes are still competent for fusion when given the appropriate signals. The fact that levels of mononucleates have not returned completely back to that of the control, is likely to be the result of only allowing the cells 24 hours to fuse when the original culture was given three days. It may also be the case that given the fairly dramatic cell movements that have taken place during treatment, some of the mononucleates are no longer in an environment, relative to other cells, that is favourable for fusion, after all, even in an untreated culture, only around one-third of the cells actually undergo fusion to form myotubes as shown in Table 3-4. Although the assay does not directly rule out the possibility that the MHC positive mononucleates produced by 109 are not viable and simply died during the 24 hour period following 109 treatment, this seems unlikely. There were no obvious signs of cell death in the culture and earlier assay data showed that the percentage of MHC positive mononucleates doubled if the period of 109 treatment was increased from 24 to 48 hours. If the cells were dying you would expect to see a decrease in the levels of MHC positive mononucleates or at least a plateau. More definitive data on cell viability and characterisation of the cells following removal of 109 is shown in later chapters of this thesis.

The dependence of the action of 109 on *de novo* protein synthesis was tested in order to provide an indirect indication of the importance of changes to gene expression. The original report of myoseverin described a distinctive gene expression profile in response to the compound, implying that, in conjunction with the observed cellularisation, myoseverin may be activating a programme characteristic of that involved in urodele

regeneration (Rosania et al., 2000), the dependence, or otherwise, of the action of myoseverin on protein synthesis, would help to determine the significance of these gene expression changes. The assay results presented in this chapter, with 109 in the presence of protein synthesis inhibitors, are a little difficult to interpret, one issue being that protein synthesis inhibition is not 100%. Maximal inhibition with 10μM anisomycin is 93% although this is probably an under-estimation of the true level of inhibition as despite washing of the cells to remove any free radioactive leucine, it is possible that some traces will remain as labelled aminoacyl-tRNA, or metabolites, although leucine was chosen in order to minimise the latter possibility. Higher concentrations of inhibitor were originally tested on C2C12 cells and found to cause increasing levels of cytotoxicity. Use of the RNA inhibitor actinomycin D to try and clarify the situation was unsuccessful as the compound was toxic to cells at all concentrations tested. The intermediate levels of fragmentation achieved by 109 in the presence of anisomycin or cycloheximide suggest that protein synthesis is not absolutely required, as some cellularisation still occurs at all concentrations of inhibitors, but the fact that the action of 109 is significantly inhibited (up to 69%) indicate that protein synthesis greatly facilitates the process.

However, the fact that some low level of protein synthesis is perhaps still occurring means that the dependence of the process of cellularisation on protein synthesis may be much greater than just suggested, and the limited protein synthesis still taking place could account for the limited amount of cellularisation. The appearance of the culture after treatment with 109 in the presence of 10μM anisomycin, showing several myotubes apparently in the process of fragmentation, allows one to propose a number of possible scenarios. It may be the case that in the absence of protein synthesis (or with only limited synthesis occurring), fragmentation can still occur but happens more slowly (this possibility was not tested because of concern that extended periods of protein synthesis inhibition would begin to affect cell viability), alternatively fragmentation may proceed but for only a limited time until the stores of necessary protein within the myotube are exhausted and the requirement for *de novo* synthesis becomes the rate-limiting step. A final possibility is that myoseverin/109 has targets in the cell other than microtubules, and is able to influence gene expression to bring about cellularisation. One model could be that the microtubule depolymerising action of 109 is protein synthesis independent,

but required to alter the myotube morphology to produce 'weak points' along the length of the myotube i.e. regions where two parts of the cell are only connected by a narrow cytoplasmic bridge. This would account for the obvious changes in cell shape seen on treatment of 109 plus anisomycin. The actual physical process of fission would then be completed in a protein synthesis dependent manner by a protein(s) whose expression is regulated by 109.

Even if 109 does not have an alternative target there can be little doubt that changes in the cytoskeleton will themselves bring about changes in gene expression which may then have a significant impact on the process of cellularisation (the effect of microtubule depolymerisation on gene expression is discussed in later chapters). While these results leave some ambiguity over the nature of the dependence of the action of 109 on *de novo* protein synthesis, they do provide evidence that decreasing levels of protein synthesis within the cell has a significant effect on the ability of 109 to bring about cellularisation of myotubes. This argues against cellularisation as a process entirely independent of the synthesis of new proteins and suggests that further investigation of changes in gene transcription (in addition to preliminary work carried out by Rosania et al) may have the potential to shed light on the mechanism of action of 109 and the more global significance of cellularisation in the context of regeneration.

Finally, in this chapter, I have clearly shown cellularisation of individual myotubes using time-lapse microscopy and cells stably expressing a nuclear localised eGFP protein. Purification of myotubes reduced the complexity of the culture while still maintaining the morphology of individual myotubes, and time-lapse microscopy allowed the capture of cellularisation events in real time, a quality that other experimental approaches do not possess. Merely imaging cells before and after fragmentation can allow cellularisation to be inferred but lacks the depth of information obtained from imaging by time-lapse. The movie included here shows nuclear migration, cytoplasmic constriction and finally resolution of these constrictions to form cells with a single nucleus. Such convincing evidence of cellularisation events, leading to the generation of mononucleates from a myotube whose terminally differentiated characteristic is a multinucleate state, has not previously been demonstrated.

Chapter 4

The role of microtubules in cellularisation

4.1 INTRODUCTION

The only known cellular target of myoseverin and 109 is microtubules. The demonstration of cellularisation of single myotubes by time-lapse microscopy has allowed closer observation of the morphological changes occurring in response to 109, and provides the grounds for a more in-depth investigation of the role of microtubules in the process. Indeed, it is difficult to imagine how such events could occur without dramatic rearrangement of the cytoskeleton. Microtubules take part in many vital processes within the cell, and changes in microtubule dynamics are known to be a driving force in bringing about morphological changes (Bulinski and Gundersen, 1991; Vega and Solomon, 1997). Myogenic differentiation involves striking morphological changes, with concomitant rearrangement of the microtubule cytoskeleton. The rearrangement of microtubules from a radial to a linear array (Musa et al., 2003; Tassin et al., 1985; Warren, 1974) plays an important role in the differentiation of myoblasts participating in the elongation and alignment of the cells prior to fusion. It therefore also appears likely that microtubules may be involved in morphological changes which denote dedifferentiation events, and that changes to microtubule dynamics have an important role in bringing about cellularisation in response to 109. This chapter aims to investigate the role of the cytoskeleton, and more specifically, to elucidate the significance of changes to the microtubule cytoskeleton in the process of cellularisation of mammalian myotubes by 109.

The first half of this chapter uses comparison of 109 with other known microtubule binding molecules to look for evidence of a correlation between microtubule-binding activity and the ability to fragment mammalian myotubes. Both the effect on organisation of the cytoskeleton, as judged by staining of actin and microtubule networks, and their activity in a previously characterised cellularisation assay, is considered. In the second half of the chapter the application of microarray technology takes these findings further and describes regulation of gene expression in response to depolymerisation of the microtubule cytoskeleton and cellularisation.

Since its conception over a decade ago, microarray technology continued to develop (Lockhart et al., 1996; Schena et al., 1995) and now enables researchers to monitor the expression of thousands of genes in response to a given condition or stimulus. Gene

chips have been used to characterise processes as complex as ageing (Lee et al., 2000a; Pletcher et al., 2002) and differentiation (Shen et al., 2003), to build up profiles of disease states (Chen et al., 2003; Porter et al., 2002) and in some cases to shed new light on cellular responses previously thought to be well characterised. This was the case for investigators at Stanford University who applied microarray technology to profile the response of fibroblasts to serum (Iyer et al., 1999). The process had been extensively studied by other means, and was thought to be dominated by a proliferative response to mitogens in serum; the microarray results revealed that this was only part of the story and that in addition the cells were activating a programme reminiscent of their physiological role in wound healing. This study illustrates that gene chip technology can highlight the presence of a wound healing or regenerative response under circumstances previously not associated with such processes.

Rosania et al applied a similar genome-wide approach to the study of myoseverin. In this case, the process of cellularisation was previously unstudied but it was suggested that the morphological effects may reflect a significant role for the molecule as a potential therapeutic in regenerative medicine. The microarray results claimed that over 50% of the transcripts regulated in differentiated myotube cultures by myoseverin, were involved in processes such as inflammation and extracellular matrix remodelling, reminiscent of the response of fibroblasts to serum thus implicating myoseverin as a stimulus for cells to execute a transcriptional programme indicative of a cellular response to injury. The dependence of 109-induced cellularisation of myotubes on *de novo* protein synthesis, and by implication, the significance of an active transcription programme in the process, was discussed in Chapter 3. The results suggested that rather than driving the process, protein synthesis facilitated cellularisation but was not absolutely required, however, due to the incomplete nature of the block in protein synthesis some ambiguity remains and justifies further independent analysis of gene expression profiles in response to cellularisation.

Microarrays are a versatile tool and the experimental design must be carefully tailored to the specific questions being asked by each experimenter. My purpose was not to generate an exhaustive and definitive fingerprint for cellularisation, nor to create a statistically rigorous and all-embracing analysis but to look at the types of cellular processes involved in the response and to isolate potential markers of myotubes or mononucleates to further

characterise the cellularisation process. My aims in carrying out this study were to use microarrays to identify patterns characteristic of these processes in order to clarify the role of microtubule polymerisation in the mechanism of action of 109, and assess the significance of this molecule in the context of regeneration. The intended focus of this work determined the experimental design from the beginning and should be considered throughout the analysis

4.2 RESULTS

4.2.1 Comparison of the activity of 109 with that of other tubulin-binding molecules

In order to investigate the role of microtubules in the mechanism of action of 109, the activity of a number of microtubule-disrupting agents was compared with that of 109 and quantitated using the cellularisation assay developed previously. Pmi28 differentiated cultures were treated for 24 hours with 15μM 109 or with taxol, nocodazole or colchicine at a range of concentrations. Colchicine and nocodazole are both microtubule-depolymerising agents while taxol stabilises microtubules, the structures of these molecules alongside that of 109 are shown in Figure 4-1.

4.2.1.1 Induction of cellularisation by a range of microtubule-binding molecules

The percentage of MHC positive mononucleates following treatment with nocodazole and taxol is shown in Figure 4-2 as the fold change compared with parallel cultures in the absence of any compound, colchicine was toxic at all concentrations tested causing extensive cell death so its effects were not quantitated. The assay results revealed that both taxol and nocodazole were capable of bringing about cellularisation of Pmi28 myotubes, as demonstrated by an increase in the percentage of MHC positive mononucleates. Both compounds showed signs of cytotoxicity above a concentration of 500nM, which may account for the slightly erratic results with taxol showing an apparently enhanced activity at 1μM compared with higher concentrations. At 500nM, nocodazole produced a fold change in MHC positive mononucleates comparable with that for 15μM 109.

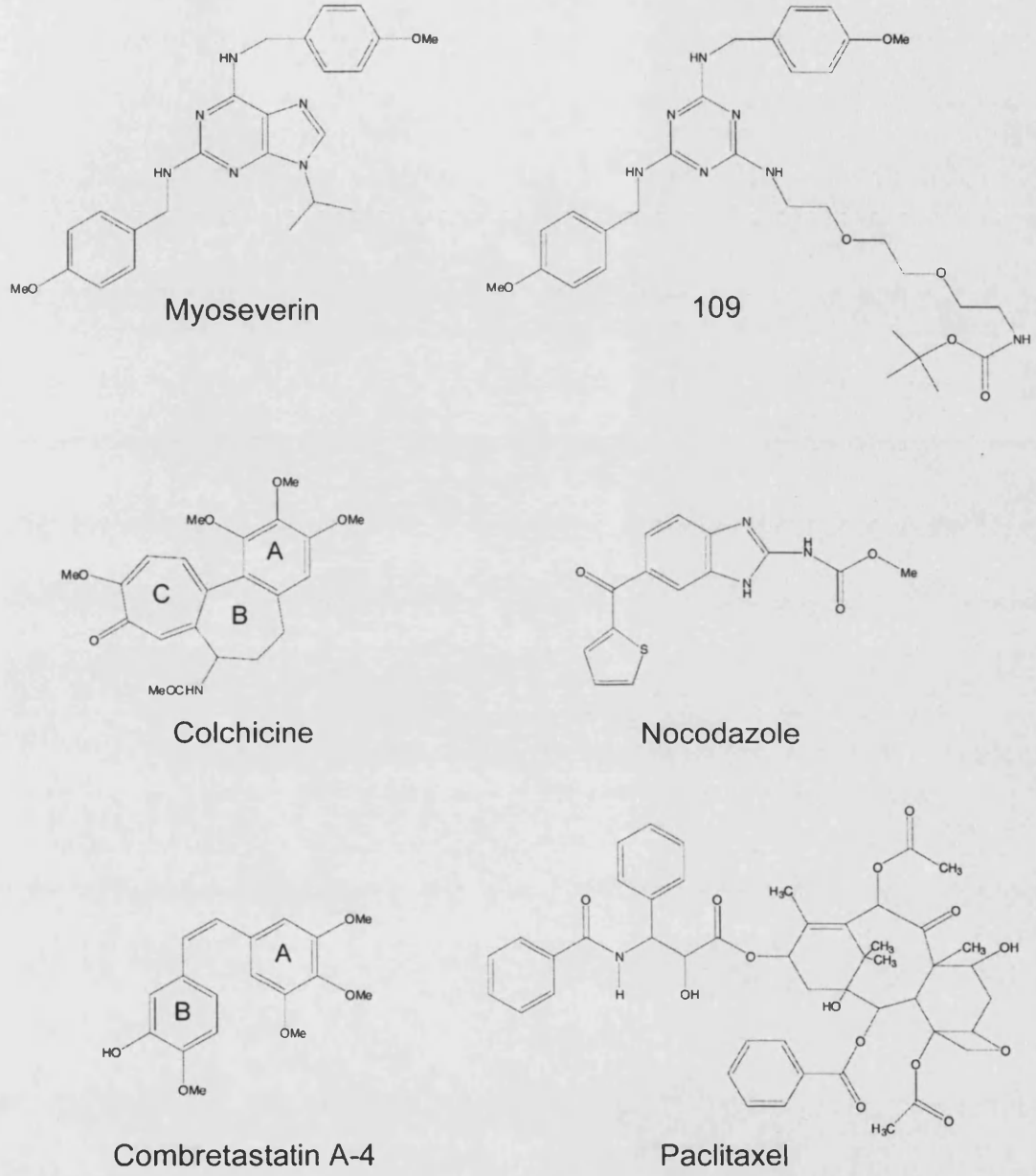


Figure 4-1: Chemical structures of microtubule-disrupting agents. Colchicine, nocodazole and combretastatin A-4 are naturally occurring microtubule-depolymerising agents. Paclitaxel, also a naturally occurring substance, is in contrast, a microtubule-stabilising molecule. Myoseverin and 109 are chemically synthesised molecules shown to bind to tubulin and induce microtubule depolymerisation. Nocodazole, paclitaxel, myoseverin and 109 were all found to have activity on Pmi28 myotubes in a cellularisation assay; colchicine was highly cytotoxic. Combretastatin A-4 was not tested.

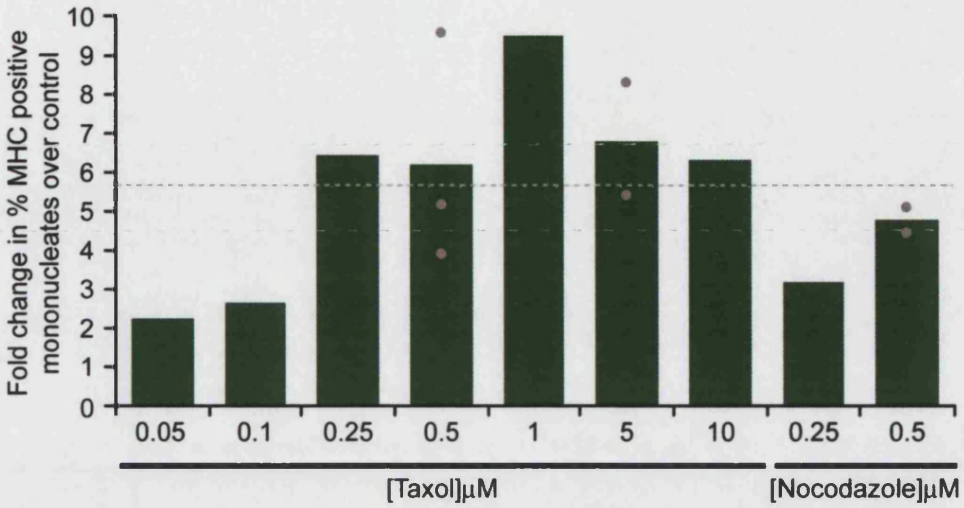


Figure 4-2: Comparison of the activity of microtubule-disrupting agents on Pmi28 myotubes in a cellularisation assay. Taxol and nocodazole were tested on Pmi28 myotube cultures for their ability to induce cellularisation. At each concentration tested, the percentage of MHC positive mononucleates was expressed as the fold change over the percentage present in a parallel untreated culture. The dashed line indicates the average for 15µM 109 (n=10). Each individual experiment incorporated counts from duplicate coverslips. Most concentrations were assayed as single experiments but note that the use of multiple concentrations reinforces the result. Where multiple experiments were carried out for a single concentration, the individual results are shown as points and the bar represents an average.

Following a period of 24 hour treatment with 109, nocodazole or taxol, myotube cultures were washed in PBS and incubated in fresh differentiation medium for a further 24 hours to establish whether the effects of the compound were reversible. Results in the previous chapter (see Figure 3-9) showed that myotube morphology is restored when 109 is removed and that the number of MHC positive mononucleates returns to a low background level suggesting that the effect of 109 on microtubules is reversible and the mononucleates produced remain competent to fuse back into myotubes. The results for all three microtubule-binding molecules are shown in Figure 4-3 as the fold change in the percentage of MHC positive mononucleates compared with no compound. All three compounds achieve comparable levels of cellularisation but while the percentage of MHC positive mononucleates reduces almost to background levels for 109 and nocodazole following removal of the compounds, the percentage remains high in taxol treated cultures after 24 hours in fresh differentiation medium.

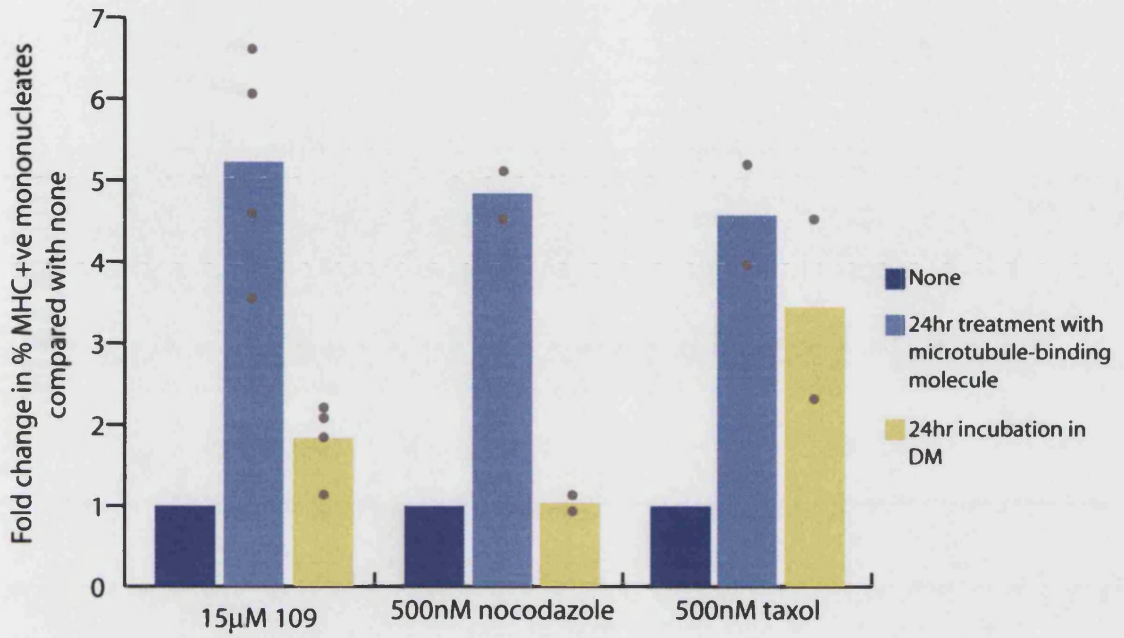


Figure 4-3: Quantitation of cellularisation in Pmi28 cultures following removal of microtubule-binding molecules. Pmi28 myotube cultures were treated with 109, nocodazole or taxol for 24 hours and the degree of cellularisation measured as the percentage of MHC positive mononucleates (as previously described in Chapter 3). Following treatment cells were then washed and maintained in fresh DM for a further 24 hours after which another measurement of cellularisation was made. While cellularisation by 109 and nocodazole appears reversible, with levels of MHC positive mononucleates returning almost to background, counts remain high in taxol treated cultures following removal of the drug. Results are expressed here as the fold change in percentage of MHC positive mononucleates. All values are relative to the percentage of MHC positive mononucleates present in a control culture treated for 24 hours with differentiation medium in parallel with the cultures treated with 109, nocodazole and taxol. In each case the control culture is represented by 1 as, for each compound, all other measurements were normalised to this value. Replicate experiments are shown as individual points while the bar represents the average.

Observations of treated cultures, fixed and stained for MHC, support differences in the reversible nature of the three compounds suggested in the assay data (Figure 4-4). Twenty-four hours after removal of 109 and nocodazole, the fragmented cultures have regained the appearance of cells prior to treatment. Cells that rounded up in response to the compounds have elongated and the majority of myotubes are long, narrow and aligned. In contrast, many of the myotubes in taxol treated cultures remain globular in appearance with nuclei clumped together rather than arranged along the length of the cell.

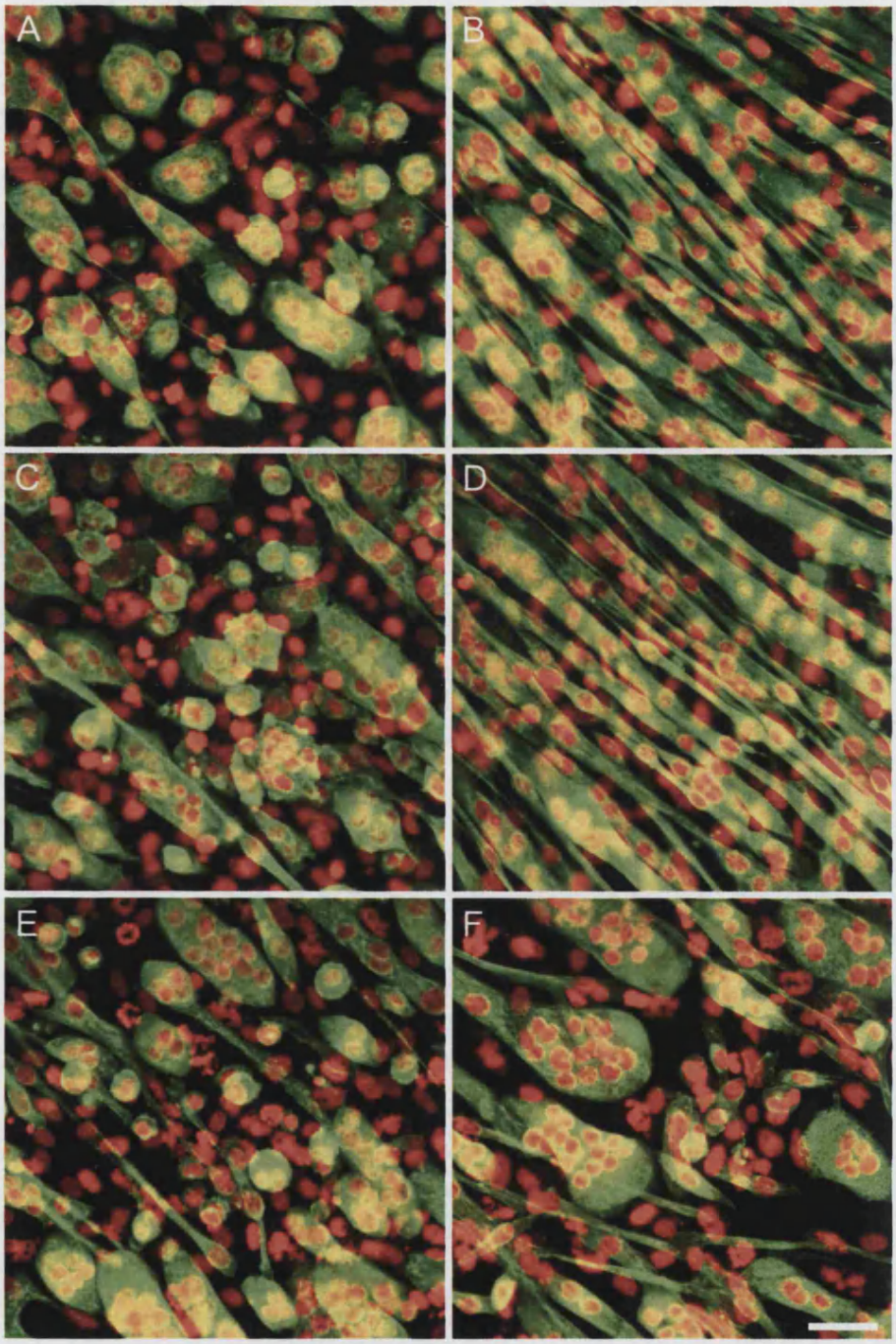


Figure 4-4: Reversibility of cellularisation induced by 109, nocodazole and taxol. Immunohistochemical staining of Pmi28 myotube cultures treated with either 109 (A and B), nocodazole (C and D) or taxol (E and F). Myotubes were exposed to the tubulin-binding molecules for 24 hours (A, C, E), after which the cells were washed and incubated for a further 24 hours in fresh differentiation medium (B, D, F). MHC staining is in green, and nuclear staining with propidium iodide in red. Scale bar = 50µm.

4.2.1.2 Examination of the cytoskeleton in treated cells

109, nocodazole and taxol treated mononucleates and myotubes were fixed and stained for cytoskeletal elements using a monoclonal antibody to β-tubulin, to detect microtubules, and FITC-conjugated phalloidin. Phalloidin is a phallotoxin that interacts specifically with F-actin. Unfortunately incompatible fixation methods excluded double staining of cells for both actin and β-tubulin.

Figure 4-5 shows mononucleate cells treated with a range of compounds and stained for β-tubulin. In the presence of 401, the microtubule network remains intact with long microtubules emanating from a region of high density (presumably the centrosome) in a radial pattern and extending in a linear fashion out to the cell cortex. The patterns of microtubule staining in 109 and nocodazole treated mononucleates are indistinguishable from one another, confirming that 109 is a microtubule-depolymerising agent. Under these conditions, the microtubules are short and broken; they do not extend far from the nucleus and the periphery of the cell is almost completely devoid of microtubules with only small fragments seen in the cytoplasm. Taxol is a microtubule stabilising agent and affects microtubule dynamics by a different mechanism to nocodazole, this is evident in Figure 4-5. The number of microtubules in the cell appears to have increased and in some regions have formed thick bundles. Although some distortion of the nucleus is often seen in mononucleates treated with 109 or nocodazole, the effect is much more pronounced in taxol treated cells, with apparent fragmentation of the nucleus.

Purified myotubes were also fixed and stained for β-tubulin following treatment with the compounds to look more closely at the mechanism by which molecules with different effects on microtubule dynamics bring about cellularisation. Figure 4-6 shows myotubes caught in the process of cellularisation after a 24 hour treatment period. In control myotubes, microtubules are aligned along the length of the cell. In both 109 and nocodazole treated myotubes, the microtubules are much less organised with no clear orientation. Although the integrity and density of the microtubules in the main body of the myotube is not as dramatically affected as in the mononucleate cells, there is significant depolymerisation in the region between two nuclei corresponding to a narrowing of the cell at that point and invagination of the cell membrane.

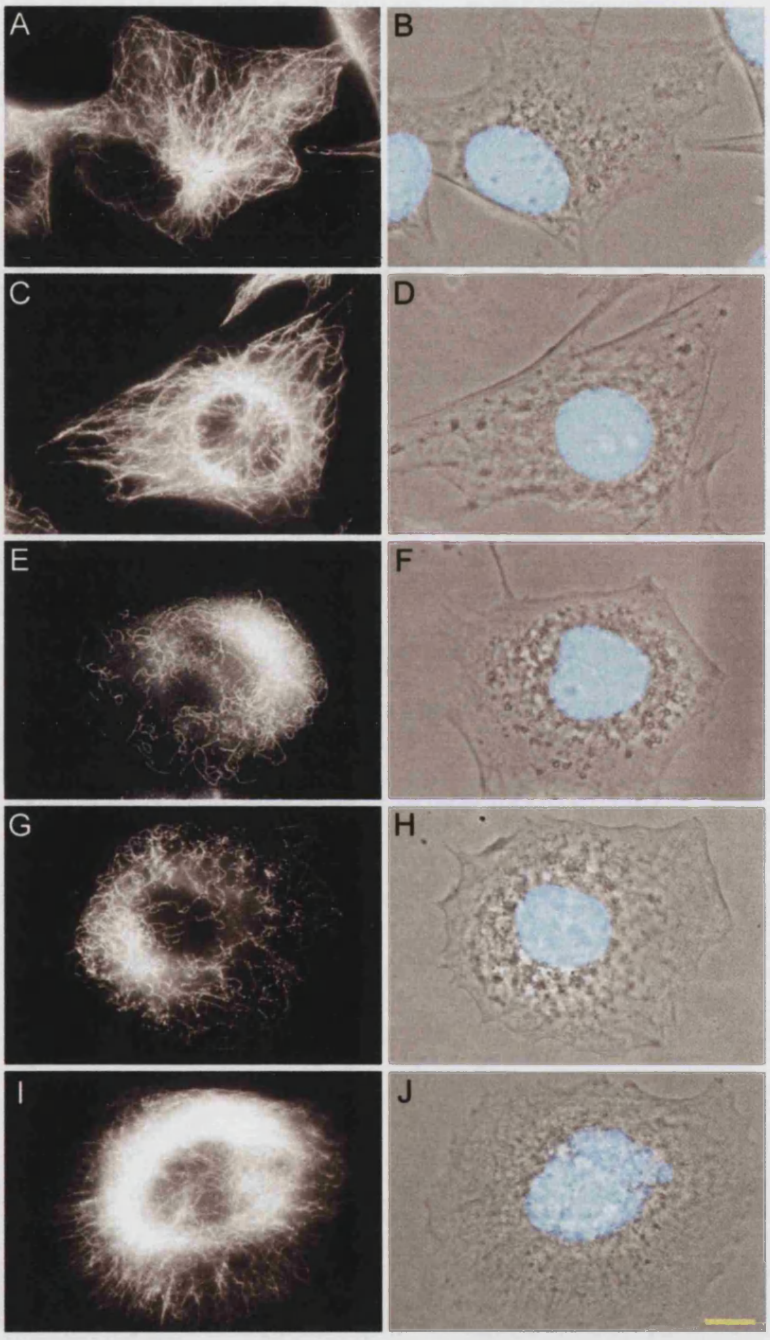


Figure 4-5: Visualisation of the microtubule cytoskeleton in treated Pmi28 mononucleates. Fluorescence and phase contrast images of Pmi28 mononucleates following treatment with DM alone (A, B), 15µM 401 (C, D), 15µM 109 (E, F), 500nM nocodazole (G, H) or 500nM taxol (I, J). Cells were fixed and stained with a monoclonal antibody against beta-tubulin (A, C, E, G and I); nuclei were stained with hoechst (in blue, overlaid with phase contrast images). No effect is seen in the presence of the inactive compound 401; a radial array of microtubules extending out to the edge of the cell is visible. In the presence of both 109 and nocodazole, the microtubules have become fragmented and disorganised while the microtubule-stabilising agent taxol appears to cause an increase in the mass of tubulin polymer and characteristic microtubule bundling around the nucleus. Scale bar = 10µm.

Again, the difference in the mechanism of action of taxol reveals a microtubule staining pattern distinct from that seen with depolymerising agents. Taxol also induces cellularisation of myotubes but sites of cellular invagination correspond to localised patches of cytoplasm devoid of microtubules, while in the rest of the cell microtubules remain aligned along the length of the cell and appear to be at higher density than in control cells.

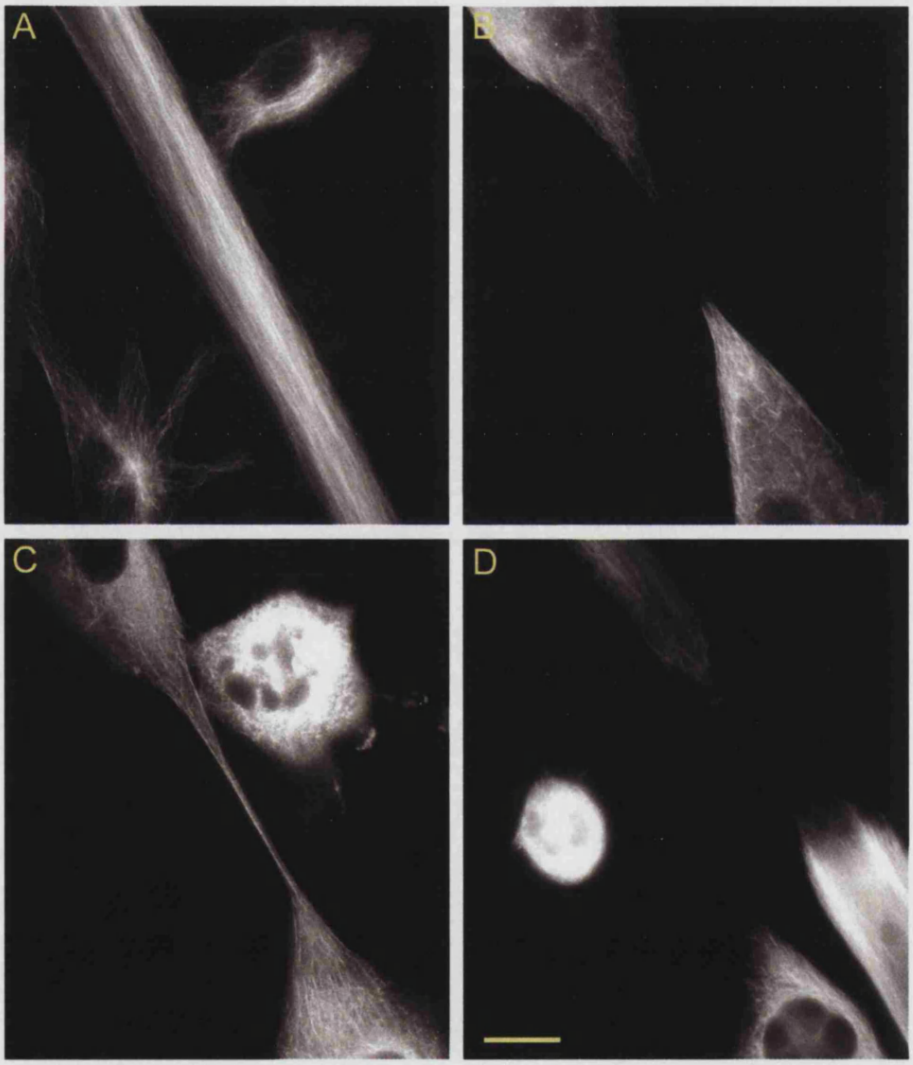


Figure 4-6: Immunohistochemical staining of microtubules in myotubes undergoing cellularisation. Pmi28 myotubes were treated for 24 hours with medium alone (A), 15μM 109 (B), 500nM nocodazole (C) or 500nM taxol (D) then fixed and stained with a monoclonal antibody to beta-tubulin. Myotubes in the process of undergoing cellularisation were identified, by the presence of a narrow cytoplasmic bridge joining two multinucleate fragments, and photographed. Scale bar = 20μm.

Despite dramatic effects on the microtubules in response to 109, Figure 4-7 demonstrates that there in no perturbation of the actin cytoskeleton. In control cells, actin filaments are aligned, this pattern remains in 109 treated cells with actin staining extending to the cell

cortex even in the presence of a severely disrupted microtubule network. Attempts to depolymerise the actin cytoskeleton with latrunculin A, to determine the effect on microtubules and on cellularisation, were unsuccessful due to the highly toxic effect of latrunculin A on cells, and especially on myotubes, at concentrations sufficient to induce depolymerisation.

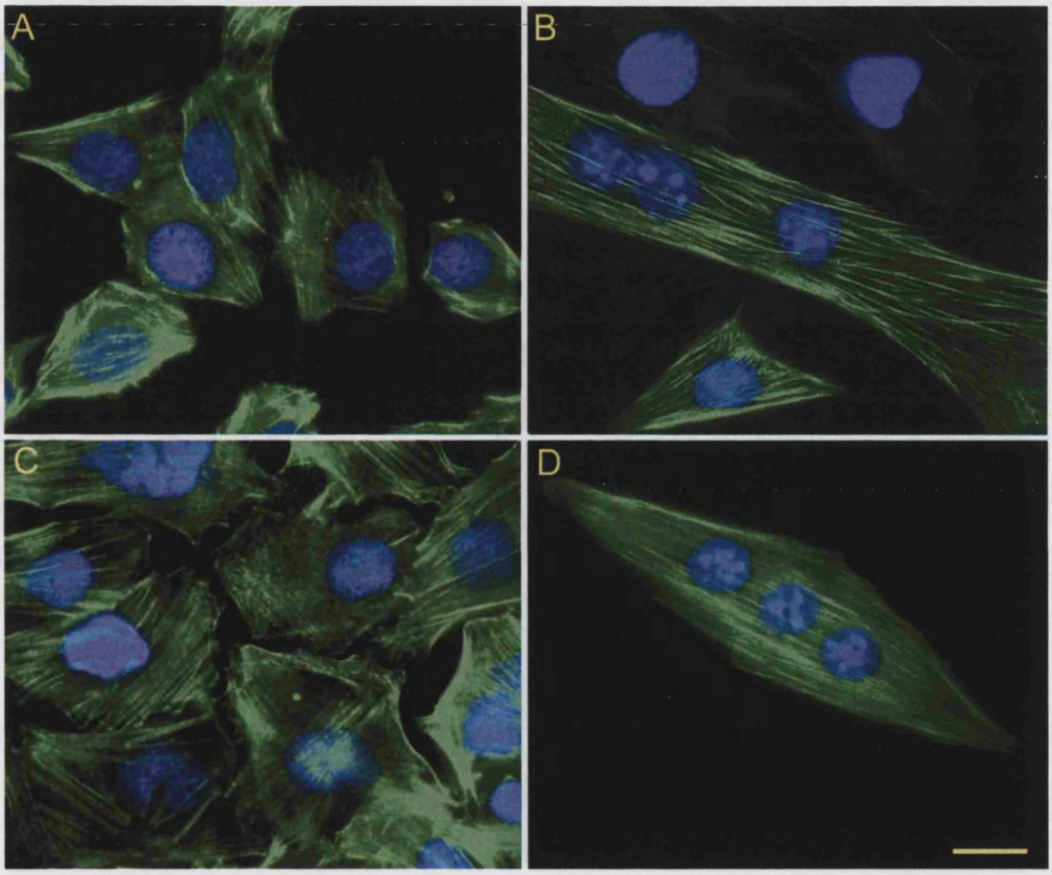


Figure 4-7: The effect of 109 on the organisation of the actin cytoskeleton. Immunohistochemical staining of control (A, B) and 109 treated (C, D) Pmi28 mononucleates and myotubes, with FITC-conjugated phalloidin (green) and hoechst (blue). In comparison to the striking changes in microtubule organisation on treatment with 109, the actin cytoskeleton within the cell remains intact. Scale bar = 20μm.

4.2.1.3 Demonstration of cellularisation by nocodazole at a single cell level

Nocodazole appears to mimic 109 in its ability to cellularise myotubes in a reversible manner, and in its direct effects on the microtubule cytoskeleton. In order to examine the specific morphological changes taking place in response to this molecule, purified Pmi28 myotubes were treated with nocodazole and the events of cellularisation observed by the use of time-lapse microscopy. Selected images from a time-lapse movie (see video3 in Supporting Data) are shown in Figure 4-8 and demonstrate the stages of cellularisation. There is initial narrowing of the myotube on either side of the nucleus, beginning almost

immediately, until the cell remains attached by only thin cytoplasmic bridges; first one and then the other of these bridges breaks leaving a single mononucleate cell. These events recapitulate those observed during cellularisation of myotubes by 109 as can be seen by comparison of Figure 4-8 with Figure 3-14. Examination of cellularisation by 109 and nocodazole at the cellular level shows the two molecules to be indistinguishable. These similarities support a significant role for microtubules in the process of cellularisation

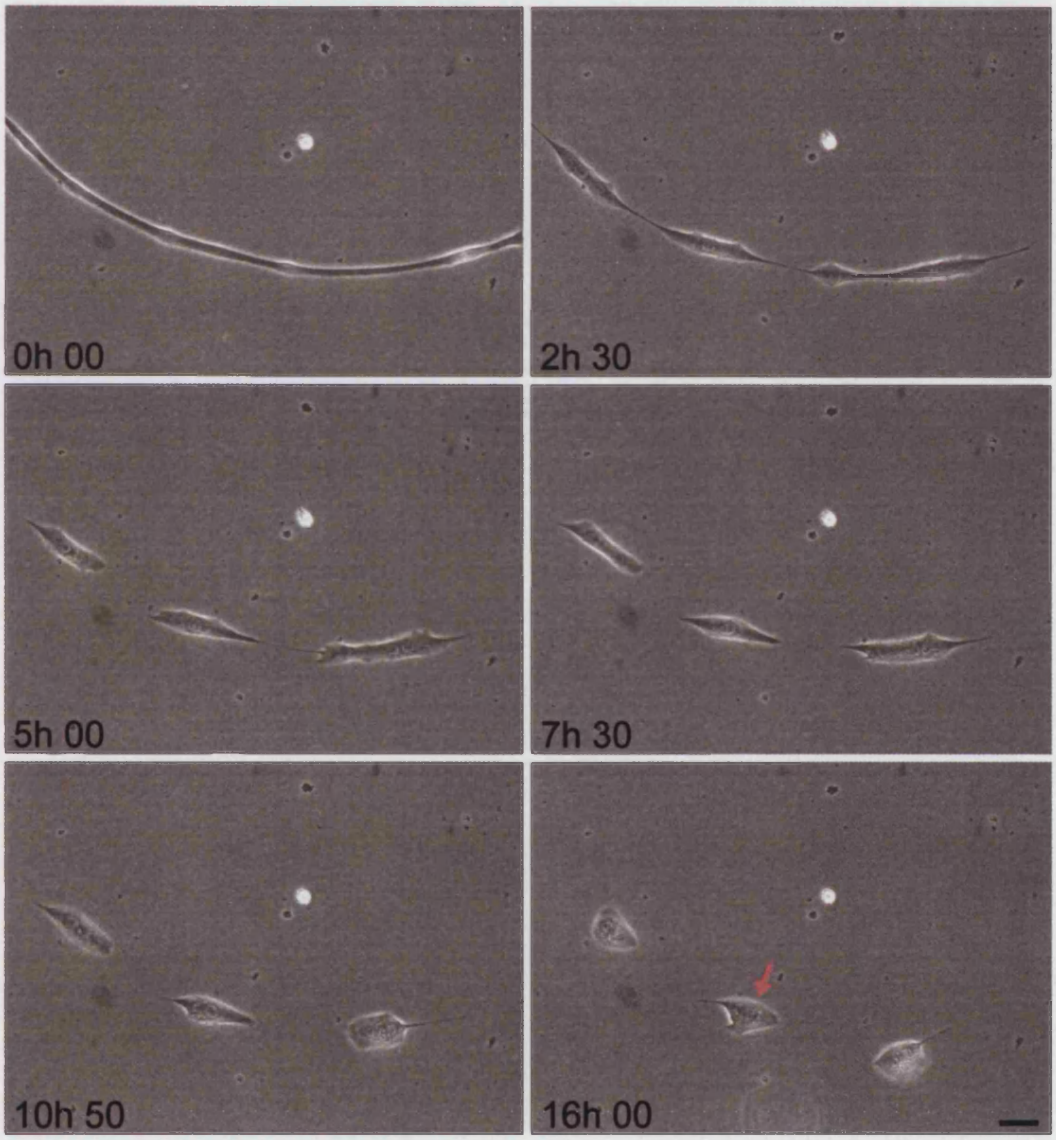


Figure 4-8: Demonstration of cellularisation of Pmi28 myotubes by nocodazole at a single cell level. Pmi28 myotubes were purified and individual myotubes imaged by time-lapse microscopy over a 16 hour period of treatment with 500nM nocodazole. Selected frames from a time-lapse sequence are shown here to illustrate the progression of cellularisation. The arrow indicates the resulting mononucleate cell. Scale bar = 50µm.

4.2.2 Examination of microtubule-depolymerisation and cellularisation at a molecular level by microarray analysis

As the effects of nocodazole and 109 appear to be analogous at this level, I have subsequently taken a molecular approach to further characterise the process of cellularisation and study the mechanism of action of these two molecules. The findings with nocodazole can be exploited to dissect the microtubule-binding properties of 109 from any other effects of the molecule, thus helping to isolate gene expression changes unique to 109 and assess their significance in the context of the hypothesis, proposed following the discovery of myoseverin, that 109 may be activating a regeneration-like programme in mammalian cells.

4.2.2.1 Analysis of microarray data

RNA was extracted from 10cm dishes of Pmi28 differentiated cultures treated with either 15μM 109, 500nM nocodazole or 15μM 401 for between 0 and 24 hours. A total of twenty RNA samples were extracted, processed and hybridised to twenty separate Affymetrix gene chips (see Figure 4-9). The MG-U74Av2 array contains over 12 000 probe sets representing around 6 000 sequences from the mouse Unigene database and a further 6 000 ESTs. This chip superseded the Mu6500 chip, which was used in the original analysis with myoseverin and contained only half the number of sequences.

Following hybridisation, washing, staining and scanning of each chip, initial expression measurements were made using Affymetrix Microarray Suite 5.0. The data files were then analysed using the GeneSpring software. The expression data for 109, nocodazole and 401 chips was normalised and filtered as described in Materials and Methods to leave only probe sets which had undergone a statistically significant fold change of greater than 1.5-fold during the 24 hour experiments. A p-value cut-off of 0.05 was used for all statistical tests. Post-hoc testing was carried out to determine between which timepoints the significant fold-change had occurred and so categorise each gene as up- or down-regulated by at least 1.5-fold during the 24 hour period.

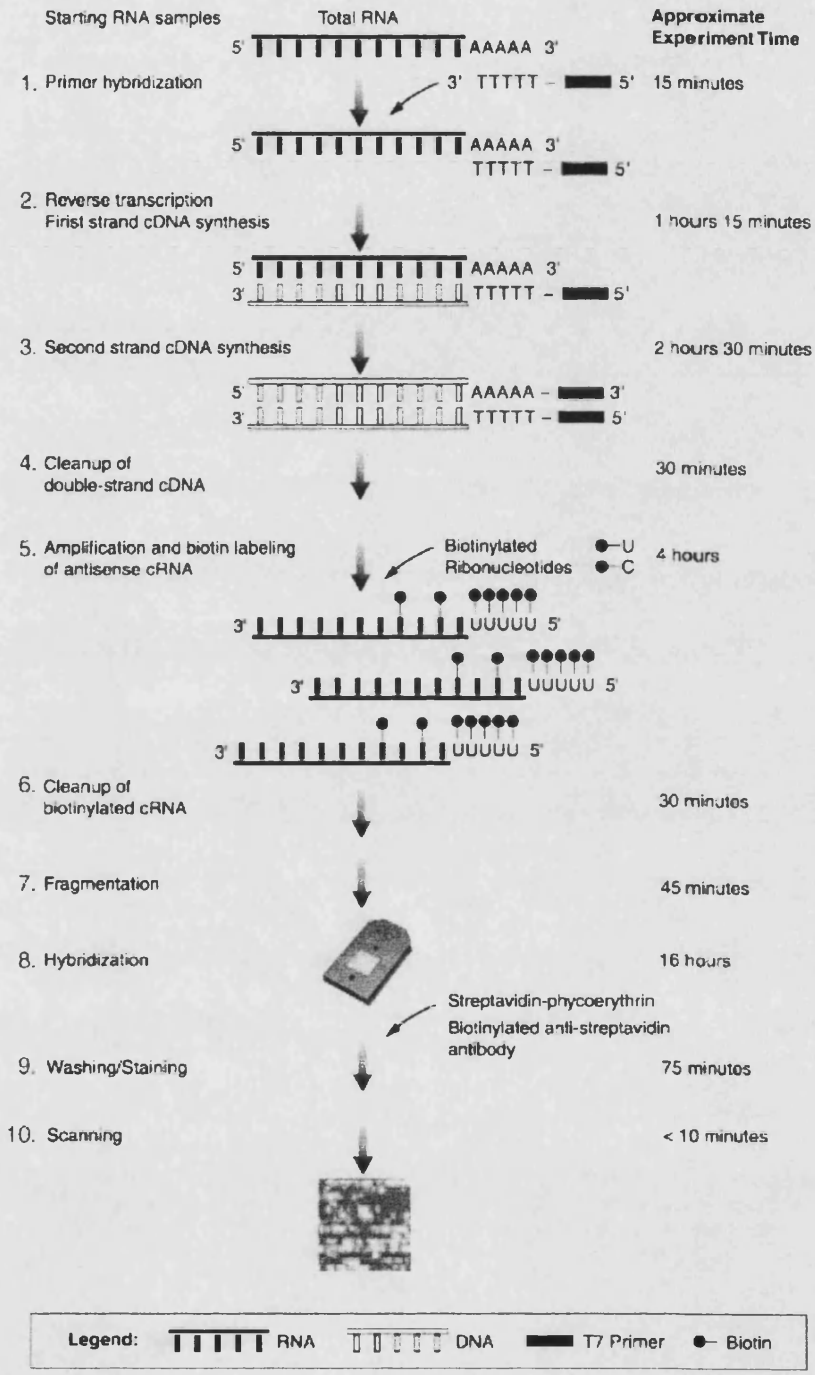


Figure 4-9: Schematic of microarray experimental procedure. Total RNA was extracted from Pmi28 myotube cultures at various time points following treatment with 109, nocodazole or 401. DNA was synthesised using an oligo dT primer incorporating an RNA polymerase promoter. An in vitro transcription reaction was carried out using RNA polymerase, in the presence of biotinylated nucleotides, and the resulting labelled cRNA fragmented for hybridisation to Affymetrix MG-U74Av2 arrays. The Affymetrix system involves the hybridisation of one sample to one array chip. Following hybridisation, each chip was passed through a series of wash cycles then stained, first with streptavidin-phycoerythrin , then with anti-streptavidin, biotinylated antibody and finally again with streptavidin-phycoerythrin to amplify the signal. The signal was scanned by a confocal laser with the read-out providing a measure of the relative abundance of each transcript. (Taken from: www.affymetrix.com).

4.2.2.2 Summary of the numbers of genes altered

Figure 4-10 shows a non-redundant summary of the number of sequences altered with each individual compound and those that were altered in more than one condition. A total of 91 genes were altered by 109, 114 by nocodazole and 75 sequences were altered by 401. The numbers include both known genes and ESTs. Full, annotated lists of all differentially regulated genes can be found in Supporting Data.

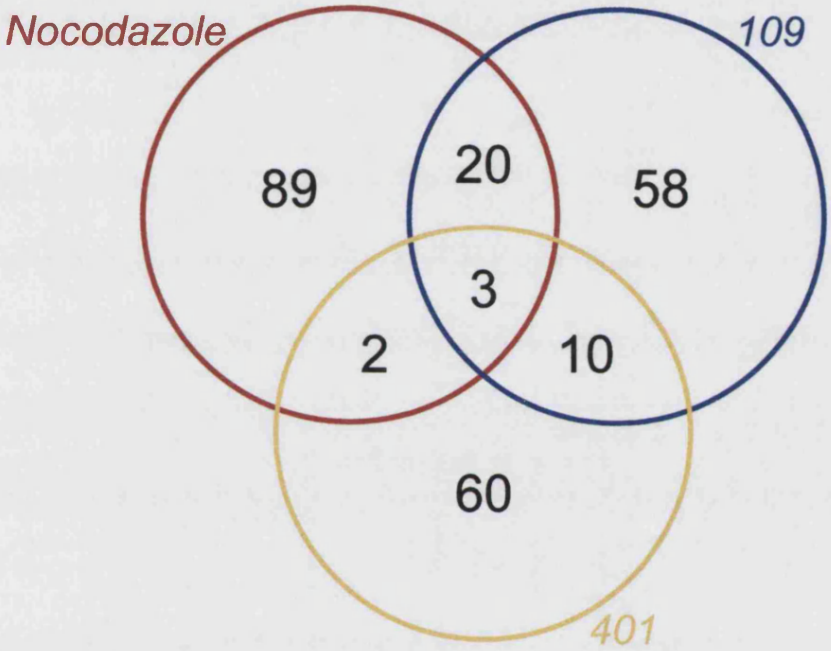


Figure 4-10: Summary of genes altered >1.5-fold in response to 109, nocodazole or 401. A total of 242 independent genes were significantly altered by at least 1.5-fold, with at least one of the compounds. Both up- and down-regulated genes are included in the Venn diagram, which displays the numbers of genes regulated by more than one compound.

The greatest number of transcripts is altered by nocodazole, a compound known to have a wide range of effects on cells due to its microtubule depolymerising properties while the smallest number of genes is altered by 401 which is inactive in the cellularisation assay and does not bind microtubules. The largest group of genes regulated by more than just one molecule is that for 109 and nocodazole. Both molecules have been shown to bind to microtubules and bring about cellularisation; the set of sequences altered significantly by both compounds accounts for 25% of the total number of genes altered with 109, and 20% of those altered with nocodazole.

Only three genes are altered with all three molecules which, given the inactive nature of 401, may represent processes which take place in the culture over time and occur independently of the microtubule cytoskeleton. C-fos and Sox-11 are transcription factors while Sca-1, the only one of the three to be up-regulated, is a GPI-anchored protein with largely unknown function. All three have been reported as up-regulated during early myogenesis in C2C12 cells. As may be expected, this group of genes is small; interfering with the cytoskeletal network is likely to have an impact on many aspects of cellular activity including cell growth, differentiation, metabolism and transport.

There are very few genes regulated by 401 and nocodazole, the two molecules share neither a common activity nor high structural similarity. Interestingly, the number of sequences shared by 109 and 401, two molecules which are almost structurally identical, is greater than that shared by nocodazole and 401. This may suggest that nocodazole is having a more dramatic or widespread effect upon the cells than 109.

4.2.2.3 Functional annotation

The next stage of analysis was to assign functional data to the gene lists to enable characterisation of the types of genes and processes involved, within a biologically relevant context. A common strategy employed to deal with the large numbers of genes generated by microarrays and aid interpretation of the effects of the compounds tested on cellular processes, is to group genes based on their function and/or location in the cell. Categorisation of genes regulated by 109, nocodazole or 401 was carried out using the Netaffx Analysis Centre.

The Netaffx Analysis Centre (Liu et al., 2003) is a freely available online tool provided by Affymetrix to complement the Affymetrix GeneChip system and assist in the interpretation of data from microarray experiments, it is available via the Affymetrix website (http://www.affymentrix.com/analysis/). It contains detailed information on the sequences and source of all probes within a probe set on a given chip, and integrates information from many public domain databases providing links to LocusLink and Genbank accession numbers. Netaffx enables the user to input a list of interesting probe sets and then query this data set to allow grouping of the data. Of particular relevance

when trying to interpret data in a biologically meaningful context is the inclusion of information from the Gene Ontology (GO) project (http://www.geneontology.org). The GO project is a collaboration, which aims to describe gene products in terms of their biological processes, molecular functions and cellular components (Ashburner et al., 2000). Where GO terms are available for a given probe set (representing a gene product) these are integrated into the Netaffx database.

I chose a series of search terms with which to query my data set through the Netaffx Analysis Centre. Although many of the terms were found within the GO annotations, I expanded my search to include all fields in the Netaffx probe set records to try and avoid missing poorly annotated sequences. As a single gene product may have more than one molecular function, be involved in several biological processes and be associated with more than one cellular component, some genes appear in more than one category.

The search terms used to categorise the genes were chosen, based on cellular processes of interest in the context of cellularisation and regeneration. Although a more exhaustive and more numerous categorisation programme was possible, I allowed the original aims and core questions of my work to influence the direction of my analysis. Does the group of genes shared by 109 and nocodazole represent a core set of 'cellularisation genes'? Are the genes altered by 109 reminiscent of a tissue injury response, as suggested by the original work with myoseverin, and if so are such patterns unique to 109? The chosen cellular processes and the search terms used to define them are included in the first columns of Table 4-1.

The frequency of a given category within each probe set list was compared with the frequency of that category in the entire set of transcripts on the chip in order to ascertain whether certain aspects of cell behaviour were over-represented or under-represented in response to 109, nocodazole or 401. Table 4-1 shows the observed and expected number of genes in each category for the three lists, and whether any discrepancy between the two is statistically significant. None of the categories were under-represented but several contained a greater number of sequences than predicted.

Table 4-1: Functional annotation and enrichment of categories.

Gene category	Search terms	109		Nocodazole		401		Array
		Obs	*Exp*	*Obs*	*Exp*	*Obs*	*Exp*	*Obs*
Muscle	muscle	15(13)	10.73	**28(24)**	**13.77**	9 (5)	9.22	1457
Cell cycle	cell cycle	**9 (1)**	**3.49**	7 (6)	4.48	6 (5)	3	474
Transcription	transcription	12 (3)	9.1	14 (4)	11.67	12 (7)	7.81	1235
ECM	extracellular matrix	3 (2)	1.51	2 (2)	1.94	**6 (6)**	**1.3**	205
Microtubules	microtubules	**5 (1)**	**1.08**	**4 (0)**	**1.38**	2 (1)	0.92	146
Cytoskeleton	cytoskeleton; cytoskeletal	**8 (3)**	**3.59**	**13 (7)**	**4.6**	4 (2)	3.08	487
Protease	protease; proteinase; peptidase	8 (4)	5.18	8 (8)	6.64	**11(10)**	**4.45**	703
Transport	transport	8 (6)	10.68	13(12)	13.7	11 (8)	9.17	1450
Growth/proliferation	growth; proliferation	22 (6)	17.73	**33(22)**	**22.74**	**29(22)**	**15.23**	2407
Adhesion	cell adhesion	4 (1)	2.84	4 (4)	3.64	5 (5)	2.44	385
Signal transduction	signalling; signal transduction	13 (2)	9.98	18(13)	12.8	12 (6)	8.57	1355
Injury response	inflammatory; wounding; immune; stress; defence	**12 (4)**	**5.11**	9 (5)	6.56	7 (6)	4.39	694
Metabolism	metabolism	**45(20)**	**26.38**	43(27)	33.84	**34(26)**	**22.65**	3581
Cell death	cell death; apoptosis	2 (1)	1.92	2 (2)	2.46	4 (4)	1.64	260

A series of chosen search terms were used to define fourteen gene categories and the number of genes regulated greater than 1.5-fold by 109, nocodazole or 401 in each category are displayed. The actual number of genes in each category (Obs) was compared with an expected value based on the numbers of transcripts on the chip in each category (Exp). Values in parentheses represent the numbers of transcripts that were up-regulated. Comparison of the distribution of categories in each list with the distribution on the chip was carried out according to Materials and Methods and revealed that some categories were over-represented in response to a particular compound, suggesting a possible biological relationship between the compound and the cellular process described in the category. Numbers in red denote a significant enrichment.

4.2.2.4 Analysis of category groupings

Overview

The response to 109 shows a significantly increased number of genes involved in cytoskeletal organisation as does the response to nocodazole, while for 401, which does not bind microtubules, the number of genes in this category is not significantly enriched. All three compounds appear to significantly affect either cell cycle or growth and proliferation genes. The response to control compound 401 is characterised by a change in extracellular matrix expression and also by an enhanced regulation of genes broadly involved in metabolism. Specific to nocodazole is enrichment in muscle-specific transcripts while, interestingly, the response to 109 does appear to involve an enhanced number of genes with roles in mounting a response to an attack or injury. 109 is unique in regulating an enhanced proportion of genes involved in the response to injury, this group of genes accounts for 13% of the total genes differentially regulated in the presence of 109.

Genes regulated by both 109 and nocodazole

Of obvious interest in determining the mechanism of action of 109 and the role played by microtubules is the list of 23 genes regulated by both 109 and nocodazole. Table 4-2 shows the types of genes represented in this group and which categories are enriched compared to their frequency on the chip itself, the list of genes is shown in Table 4-3. Regulation of genes involved in cytoskeletal organisation and binding can clearly be seen as a significant category in this group of genes, although the actual number of genes is quite small (see Table 4-2). This observation further supports a significant role for microtubules in cellularisation. When looked at independently, all the cytoskeletal genes regulated by 109 generally indicate the down-regulation of microtubule components and binding elements, while the actin-binding profilin 2, and the muscle thin filament, tropomyosin, are up-regulated. This suggests that elements of the cytoskeleton other than microtubules are largely unaffected. The same pattern is seen in the cytoskeletal genes regulated by nocodazole, where components of the microtubule cytoskeleton are down-regulated genes while the up-regulated genes are muscle-specific thin and thick filaments or actin-binding molecules. This is in keeping with the premise that 109

affects microtubule dynamics in the same way as nocodazole, bringing about the indistinguishable patterns of microtubule depolymerisation seen by immunohistochemical staining of the cytoskeleton.

Table 4-2: Enrichment of categories with genes regulated by both 109 and nocodazole.

Gene category	109 & Nocodazole	
	Obs	*Exp*
Muscle	6 (6)	2.80
Cell cycle	1 (0)	0.91
Transcription	5 (0)	2.37
ECM	0 (0)	0.39
Microtubules	2 (0)	0.28
Cytoskeleton	3 (1)	0.94
Protease	1 (1)	1.35
Transport	2 (2)	2.79
Growth/proliferation	6 (2)	4.63
Adhesion	0 (0)	0.74
Signal transduction	3 (1)	2.60
Injury response	3 (2)	1.33
Metabolism	12 (6)	6.88
Cell death	0 (0)	0.50

The distribution of 23 transcripts, regulated in response to both 109 and nocodazole, across 14 gene categories, reveals that some categories are over-represented when compared to the distribution of the transcripts on the chip, suggesting a possible biological relationship between the shared effects of 109 and nocodazole and the cellular processes highlighted. The enriched categories are shown in red, values in parentheses represent the numbers of transcripts that were up-regulated.

A second category over-represented among the transcripts regulated by both 109 and nocodazole is that containing genes associated with muscle or involved in muscle development. All the genes in this category are up-regulated and include creatine kinase, an enzyme whose activity is often used as a measure of muscle differentiation and tropomyosin, a structural component of the muscle contractile machinery. The largest number of muscle-related genes is regulated by nocodazole; the number exceeds that expected from comparison with the chip, by a significant amount. The majority of the genes are up-regulated and include markers of the differentiation process such as myosin heavy chain, the muscle related transcription factor Myf6, and γ-sarcoglycan, all of which have been shown to be up-regulated during myogenesis.

Table 4-3: Genes regulated >1.5-fold with both 109 and nocodazole.

Accession no.	Gene name	Fold change 109	Fold change nocodazole
X04653	Sca-1	6.20	4.91
AI181132	Creatine kinase, mitochondrial 2	3.57	4.84
X70296	Serpine 2	2.88	2.74
U47737	Sca-2/TSA-1	2.80	1.97
U04541	Tropomyosin 3, gamma	2.71	3.08
M12571	Heat shock protein 1a	2.65	1.88
AJ001418	Pyruvate DH kinase 4	2.50	2.35
X83202	Hydroxysteroid 11-beta DH 1	2.08	2.09
D88994	AMP deaminase 3	2.02	1.88
L10244	Sat	1.96	2.40
X96737	Synaptobrevin like 1	1.67	1.77
AA270365	CLF-1	0.62	0.53
U61969	Wnt10a	0.57	0.47
J03750	RNA polymerase II transcriptional coactivator	0.55	0.52
X04663	Tubulin, beta 5	0.55	0.50
AA716963	Isopentenyl-diphosphate delta isomerase	0.54	0.41
AF060539	Cipp	0.53	0.36
AW122523	Fatty acyl elongase	0.52	0.39
V00727	Fos	0.51	0.46
M13443	Tubulin alpha 7	0.51	0.52
AW107922	Sox11	0.45	0.56
M33988	Histone H2A.1	0.45	0.52
M24377	Egr-2/Krox-20	0.41	0.41

A total of 24 transcripts corresponding to 23 independent genes were regulated with both 15μM 109 and 500nM nocodazole. The maximum significant fold changes over the course of the 24 hour treatment period are shown here.

Regulation of cell growth

With all three compounds, either cell cycle or growth/proliferation genes are over-represented (Table 4-1). This suggests that changes in cell cycle regulation are occurring even in the control culture, treated with 401. In the group of genes regulated by both 109 and nocodazole, 5 of the 6 genes under growth/proliferation are down-regulated by both molecules and include the proto-oncogene c-fos, early growth response factor Egr-2, and Wnt10a. Down-regulation of genes involved in the cell cycle and proliferation is also the pattern among the total list of genes regulated by 109 with only around one-quarter of the transcripts up-regulated. This seems to correlate with overall negative regulation of the cell cycle. In contrast, genes in these categories are largely up-regulated in response to both nocodazole and 401, with at least two-thirds showing an increase over the course of

the experiment. In these cases, the overall regulation of the cell cycle appears more complex with both positive and negative regulators of the cell cycle being altered. It should be noted that some of these genes might have additional functions in the cell. With nocodazole, growth response genes such as c-fos, Egr-1 and Egr-2 are down-regulated while growth arrest genes Gas2 and Gas6 are up-regulated, but also up-regulated is the growth factor FGF9 and mitogen-activated protein kinase, MAPK1.

Metabolism

Metabolism is a very broad category as evidenced by the large numbers of regulated genes in this group. The distribution of genes regulated by 109 and 401 across the gene categories reveals that of thirteen genes that are regulated by both 109 and 401, seven are involved in metabolism. The genes regulated by 401 and 109 may represent processes that occur in the culture as it ages, independently of changes to the microtubule cytoskeleton. When metabolism is looked at more closely and broken down into the types of metabolism involved, all types of metabolism regulated by 109 and 401 are also involved in the response to nocodazole. While regulation of the same genes does not necessarily occur, the same metabolic processes are taking place over time.

Adhesion and ECM

Genes corresponding to extracellular matrix components or remodelling enzymes were enriched in the list of genes regulated by 401 consistent with a role for the ECM in myogenesis (Osses and Brandan, 2002; Saitoh et al., 1992) and the continuing differentiation of the culture in the presence of 401. All genes in this list were up-regulated and include procollagen genes, fibrillin 1 and a matrix metalloproteinase, Mmp11.

A similar number of genes involved in adhesion were regulated by each of the three compounds but with 401 and nocodazole all adhesion genes were up-regulated while with 109 three of the four genes were down-regulated. All three down-regulated genes have a pro-adhesive role and include ADAM12, a metalloproteinase implicated in myoblast fusion at the onset of differentiation (Cao et al., 2003; Yagami-Hiromasa et al., 1995). The single up-regulated gene, OSF-2/periostin, has been identified as a potential regulator of skeletal muscle regeneration (Goetsch et al., 2003). All genes up-regulated

by nocodazole and 401 are pro-adhesive; CDON, a gene up-regulated with nocodazole is thought to be important for skeletal muscle differentiation providing positive feedback to MyoD (Kang et al., 1998).

Injury response

Of particular relevance to the issues surrounding a role for 109 in regeneration, is the observation that the number of genes involved in, what I have termed, 'injury response' appears to be enriched with 109 and not with either of the other molecules tested here (although both 401 and nocodazole do both regulate some genes in this category). The terms used to define this category were chosen to try and capture all genes that could be said to represent a possible aspect of regeneration, and were chosen with consideration to the original myoseverin paper, in order to try and assess the significance of their findings in my system. Of twelve genes in this category regulated by 109 (Table 4-4), six were upregulated (the two genes designated L17022 and M35247 were both significantly up- and down-regulated over the course of the 24 hour treatment, only the largest fold change is showed in Table 4-4).

Table 4-4: List of 'Injury response' genes regulated >1.5-fold by 109.

Accession no.	Gene name	Fold change 109
X04653	Sca-1	6.20
U10410	Immunoglobulin kappa chain V8	5.45
U47737	Sca-2/TSA-1	2.80
M12571	Heat shock protein 1a	2.65
L17022	Fc receptor, IgG, alpha chain transporter	2.55
M35247	histocompatibility 2, T region locus 22	1.72
U92565	CXC3 chemokine/fractalkine	0.63
U28724	Pms2	0.59
AA614971	Mail/Ikappa-zeta	0.55
M27960	IL-4R alpha	0.51
Y07519	T1/ST2	0.49
M88242	COX2	0.38

The gene category designated 'Injury response' was defined by a number of search terms based on cellular processes involved in a cellular response to injury or wounding. All genes were searched with the terms and of 91 genes regulated by 109, the 12 shown here were designated as having a role in processes such as inflammation, wounding or cellular defence.

Closer examination revealed that genes with specific roles in inflammation such as COX2, a key enzyme in the production of inflammatory mediators, and fractalkine, a

pro-inflammatory chemokine are actually down-regulated with 109. Up-regulated genes include the stem cell antigens Sca-1 and Sca-2 and a heat shock protein, which have a general role in cell defence according to their searchable GO annotation. It is noteworthy that of the six genes up-regulated with 109, the three mentioned above are also up-regulated with nocodazole.

Overall, the injury response genes regulated by 109 seem more representative of a programme of defence and cytoprotection. This hypothesis also gains some support when the list of transcripts involved in proteolysis are examined more closely. Proteases are known to be involved in inflammation and are prevalent in responses to tissue injury. Many coagulation factors and inflammatory mediators exist as inactive zymogens which require proteolytic cleavage; ECM remodelling often requires proteases and matrix metalloproteinases are a feature of inflammation, there is also the requirement for digestion and breakdown of dead cells and debris. It may perhaps seem plausible to suppose that the breakdown of myotubes into mononucleates could require protease activity.

Proteases

The genes up-regulated in response to 109 are nexin-1/serpine-2, a thrombin inhibitor, epoxide hydrolase, involved in metabolism of carcinogens and xenobiotics and serine carboxypeptidase which also appears to have cytoprotective roles. Down-regulated genes in this category include two metallopeptidases, Dpep1, which metabolises glutathione and may have a role in synthesis of inflammatory eicosanoids, and thimet oligopeptidase, which hydrolyses bradykinin, a pro-inflammatory peptide. Also down-regulated is ADAM12, a matrix metalloproteinase with a role in myoblast fusion but also in extracellular matrix remodelling and wound healing. In contrast, genes in this category regulated by nocodazole are all up-regulated but include a mix of enzymes with potentially pro-inflammatory or tissue destructive action, such as PAR-2 and trypsin 2, and those with the ability to moderate such activities, such as the thrombin inhibitor nexin-1/serpine-2 and the cysteine protease inhibitor cystatin C.

4.2.3 Choice of potential marker genes

Following analysis of the microarray data I sought to identify genes which could potentially act as markers, to further characterise and investigate the process of

cellularisation, thus taking the information gleaned from a global molecular approach and applying it at a cellular level. A potentially useful marker should ideally show a large fold change over time to give the best chance that the change will be detectable at the protein level, should be amenable to standard laboratory fixation protocols and preferably have an antibody available commercially. In addition, the marker should represent a protein or process of interest in the context of cellularisation.

Sca-1 was first characterised as a stem cell antigen and is a member of the Ly-6 family of proteins. It was identified in the microarray study as 6.2-fold up-regulated by 109 over the course of 24 hours, one of the largest fold changes seen in this study. The up-regulation of a gene associated with haematopoietic stem cells and also with a subpopulation of cells from skeletal muscle with stem cell-like characteristics, is potentially interesting in terms of evaluating cellularisation and dedifferentiation. The protein is GPI-anchored and is expressed on the cell surface; a number of commercial antibodies, most commonly applied for FACS, are available. Sca-1 was also found to be up-regulated with nocodazole and 401 by 4.91- and 4.77-fold respectively, over 24 hours.

Sca-2 is a closely related molecule and is a GPI-anchored protein of the same family as Sca-1, with overlapping, although not identical, patterns of expression in the haematopoietic system. In the microarray experiment, Sca-2 was also up-regulated, but with 109 and nocodazole only, by 2.80- and 1.97-fold respectively. The use of two stem cell antigens with different patterns of expression may prove an interesting combination, one of the genes is up-regulated in the culture over time even in the presence of the control compound while the other is up-regulated only in response to agents which bring about cellularisation.

The changes in expression levels of Sca-1 and Sca-2 in response to 109, nocodazole and 401 are shown in Figure 4-11. Although the scale is in arbitrary units reflecting the hybridisation intensities to the perfect match and mismatch sequences of the probe set on the chip, it provides a measure of the relative abundance of each transcript. Figure 4-11 shows that at the start of the experiment, the untreated cells express Sca-2 at a higher level than Sca-1.

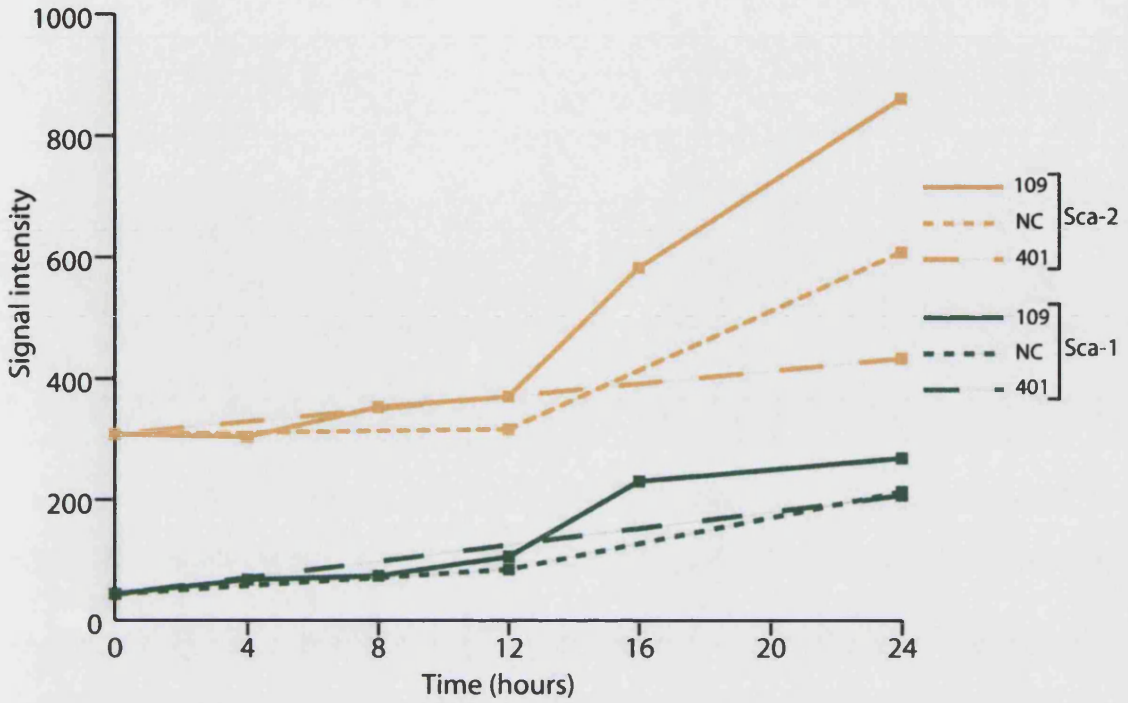


Figure 4-11: Changes in expression of Sca-1 and Sca-2 in response to 109, nocodazole and 401 from microarray analysis. Expression is represented in arbitrary fluorescence units, which provide a measure of the relative abundance of each transcript on the array. Sca-1 is significantly up-regulated by all three compounds while Sca-2 is up-regulated with 109 and nocodazole only. Although Sca-1 shows a greater fold increase over the course of the experiment, the values, taken directly from the microarray data, suggest that Sca-2 is expressed at higher levels in the Pmi28 myogenic cultures used here. For 109 and nocodazole, data points at 0, 12 and 24 hours represent an average from three independent arrays, at 4, 8 and 16 hours, points represent single determinations; for 401, data points at 24 hours are an average of two. In all cases the maximum fold change was achieved between 0 and 24 hours.

4.2.3.1 Expression of Sca-1

Pmi28 cells were stained to determine which cell types expressed the two proteins and in which populations they were up-regulated. Crucially I hoped to determine whether either of the antigens was a marker for the mononucleates newly formed during the process of cellularisation. Both antigens are reportedly expressed on the cell surface so all antibody staining was carried out on live cells, incubated on ice, with subsequent fixation by acid/alcohol. A rat anti-mouse monoclonal antibody (Pharmingen) followed by a fluorescein labelled goat anti-rat secondary antibody was used to detect Sca-1. Initially Pmi28 mononucleates were stained, the staining pattern seen was characteristic of a GPI-anchored protein, consisting of punctuate staining of the cell surface, often observed as a dotted line around the edge of the cell (see Figure 4-12, A). Staining was found to be much stronger when fluorescence microscopy was carried out with cells on glass coverslips compared with plastic culture dishes, and was much clearer when cells were incubated on ice to reduce the turnover of proteins at the cell surface during staining.

Approximately 95% of mononucleates expressed detectable levels of the protein but staining intensity varied greatly between the cells.

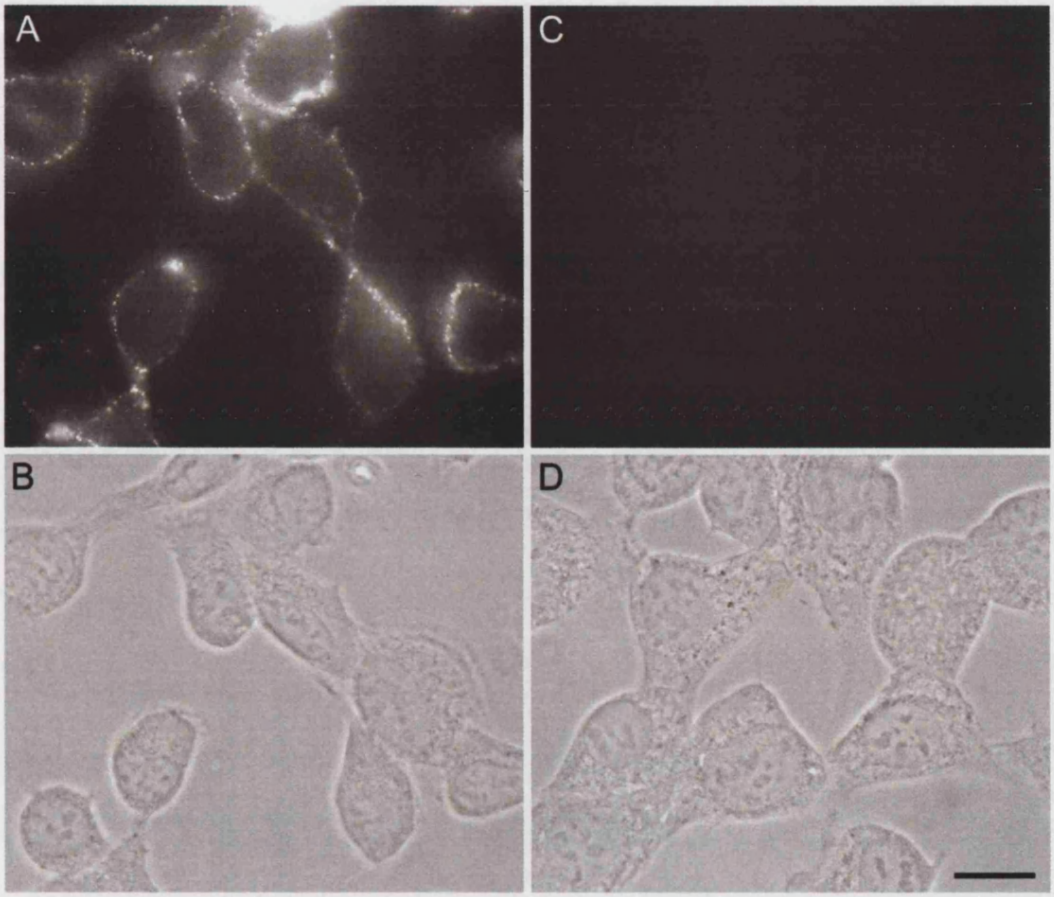


Figure 4-12: Sca-1 is a GPI-anchored protein. Proliferating Pmi28 mononucleates were treated with (C, D) or without (A, B) PIPLC for one hour to remove all GPI-anchored proteins from the surface of the cell. Following treatment, cells were stained on ice for Sca-1 and fixed. For fluorescence images of control (A) and PIPLC-treated (C) mononucleates, camera exposure times and image manipulations were identical, corresponding phase contrast images were taken to demonstrate the presence of mononucleate cells in the field. Following treatment with PIPLC, the characteristic cell surface staining pattern of Sca-1 is completely lost, demonstrating that Sca-1 is a GPI-anchored protein. Scale bar = 20µm.

Mononucleates were treated with PIPLC (phosphatidylinositol phospholipase C), which cleaves GPI anchored proteins by hydrolysing the phosphodiester bond of phosphatidylinositol. One-hour treatment with PIPLC reduced staining to almost undetectable levels confirming that Sca-1 is linked to the cell membrane by a GPI-anchor (Figure 4-12). Due to the close-packed and overlapping nature of the cultures, cell surface staining was difficult to distinguish at high magnification in the dense differentiated cultures used for the microarray analysis, so myotube purification was carried out in order to further examine Sca-1 staining of the cell populations present in a differentiated culture. Purified cells were plated onto matrigel-coated glass coverslips due to the advantages of using glass as a substrate for fluorescence microscopy in this

case. Myotube survival was low and cell morphology poor, when plated onto glass in the absence of matrigel. Sca-1 staining was clearly seen on mononucleates present after the purification process but was undetectable on myotubes, or seen occasionally as a weak non-specific staining not thought to represent true surface staining of the antigen (Figure 4-13, A and C).

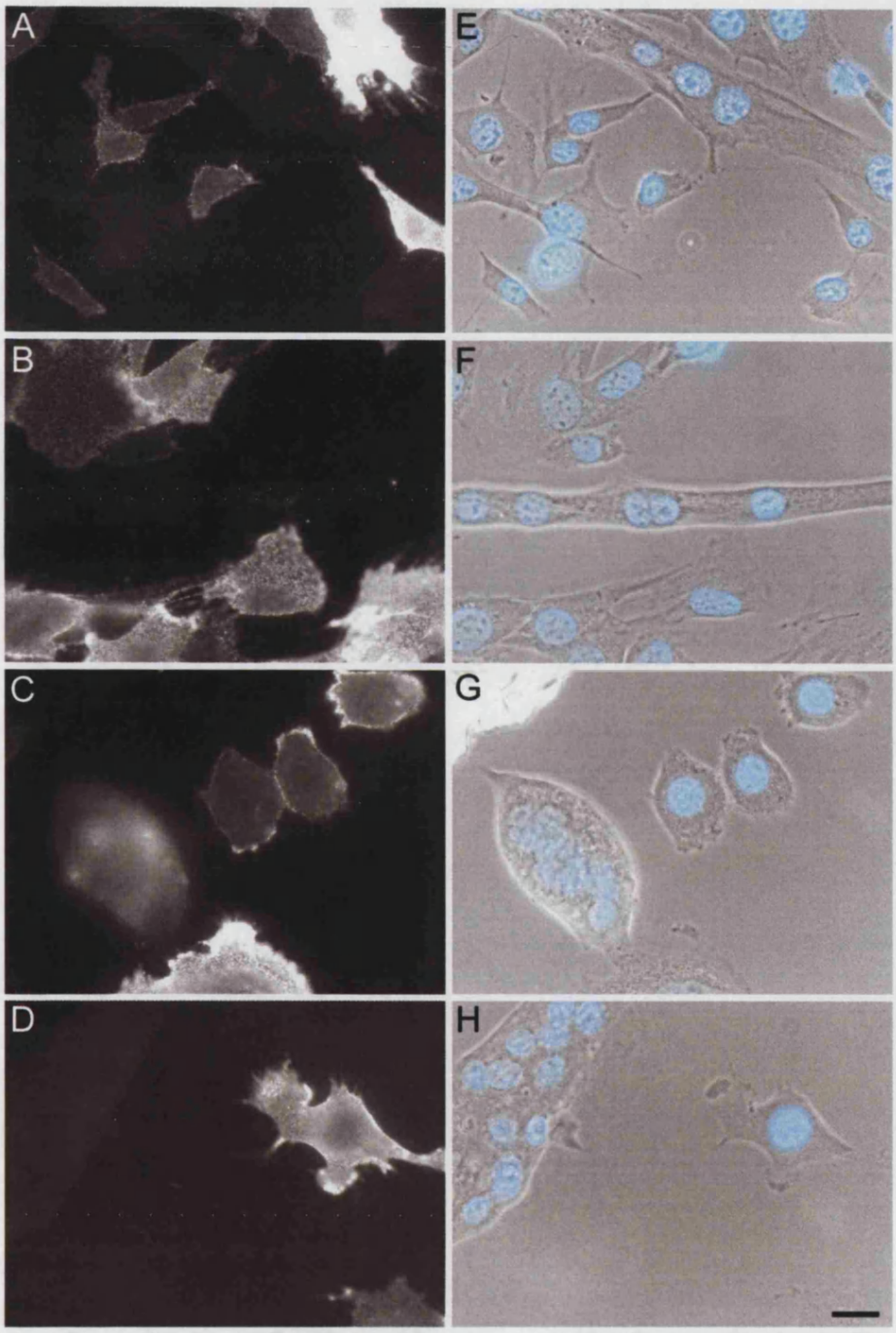


Figure 4-13: Sca-1 staining of Pmi28 myotubes treated with 109, nocodazole or 401. Purified myotubes were treated with DM alone (A), 15μM 109 (C), 500nM nocodazole (D), or 15μM 401 (B) for 24 hours followed by staining for Sca-1 on ice and fixation. Sca-1 expression was undetectable on the surface of myotubes under all treatment conditions, the majority of mononucleates in all cultures were Sca-1 positive. Scale bar = 20μm.

Double staining of mononucleates for Sca-1 followed by MHC revealed that MHC positive mononucleates appeared to be Sca-1 negative while Sca-1 positive cells were negative for MHC (Figure 4-14). It should be noted that not all Sca-1 negative cells were necessarily positive for MHC suggesting that Sca-1 staining is lost prior to MHC expression; no examples of cells staining positive for both antigens were observed.

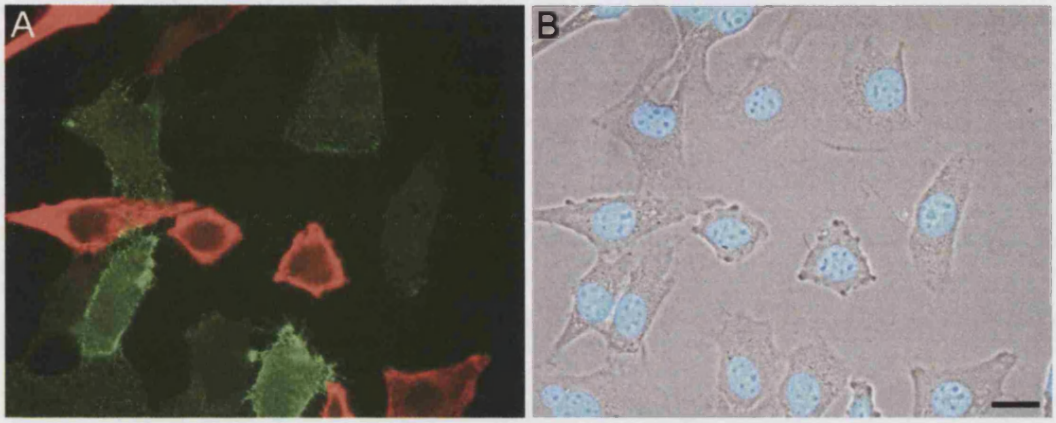


Figure 4-14: Double-staining of Pmi28 mononucleates for MHC and Sca-1. Fluorescence (A) and phase contrast (B) images of the same field of mononucleates stained for both MHC (red) and Sca-1 (green), nuclei were stained with hoechst (blue). Expression of the two proteins was never observed in the same cell. Scale bar = 20µm.

To determine whether Sca-1 is expressed by the mononucleates formed through cellularisation, time-lapse microscopy was used. Purified myotubes were plated onto matrigel-coated, glass-bottomed, 35mm culture dishes and treated with 15µM 109 for 24 hours, chosen fields were imaged at five-minute intervals. Immediately following the 24 hour treatment, cells were stained for Sca-1 and fixed as previously described. Individual mononucleates, identified from the time-lapse movies as formed by cellularisation of myotubes, were then examined for expression of Sca-1. Figure 4-15 shows selected stills from a time-lapse movie of a myotube undergoing cellularisation with 109, and the phase contrast and accompanying fluorescence image of the mononucleate produced following Sca-1 staining and fixation. The mononucleate of interest is clearly Sca-1 negative and is shown next to a pre-existing Sca-1 positive mononucleate myoblast. Two more mononucleates formed by the action of 109 on Pmi28 myotubes were Sca-1 negative. These experiments also confirmed that Sca-1 was not expressed in myotubes in response to 109 treatment.

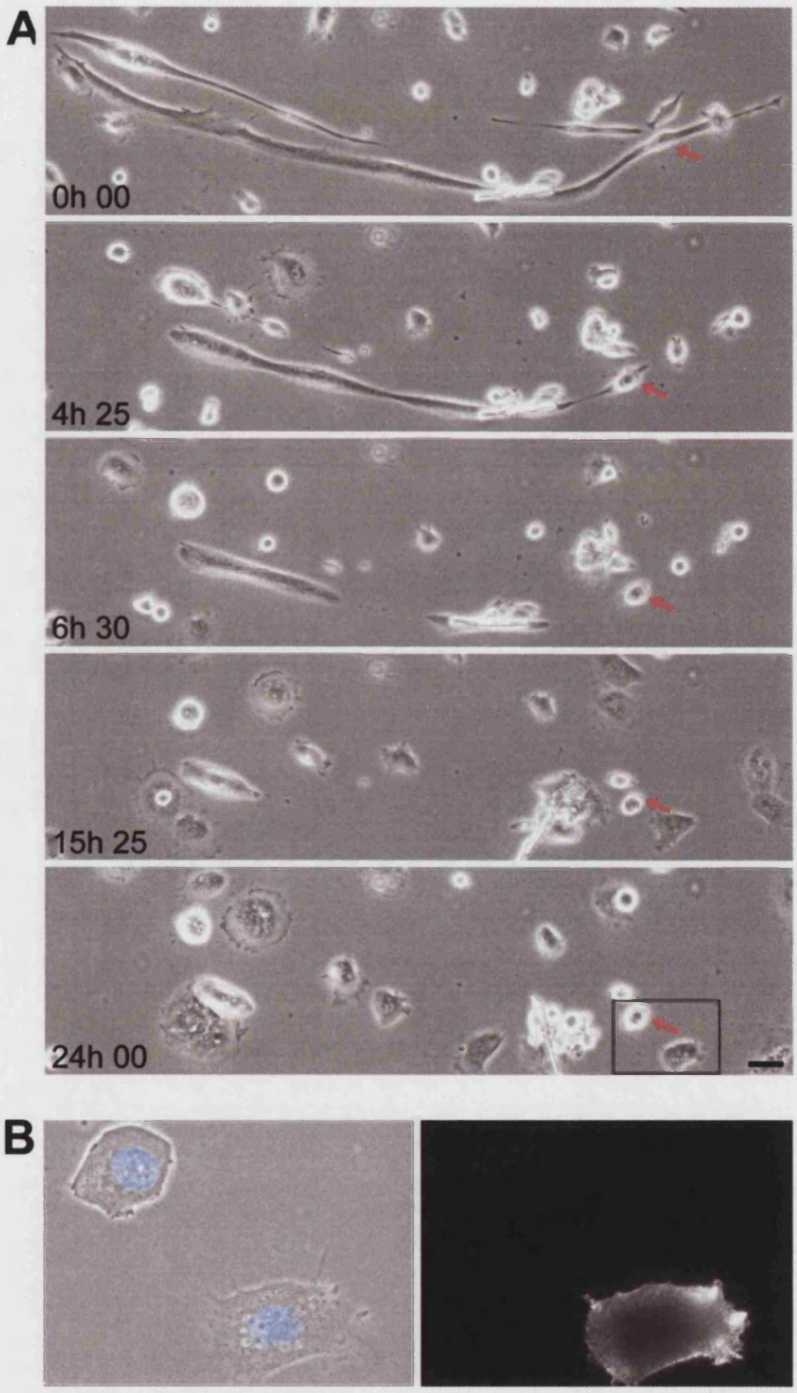


Figure 4-15: Sca-1 staining of a mononucleate progeny of cellularisation with 109. Pmi28 myotubes were purified and plated onto matrigel-coated glass-bottomed dishes. Individual myotubes were imaged over a 24 hour period of treatment with 15μM 109 by time-lapse microscopy. Selected frames from a time-lapse movie are shown here to illustrate the progression of cellularisation (A). An arrow in each panel marks the emerging mononucleate. Scale bar = 50μm. Following cellularisation with 109, cells were stained for Sca-1 and imaged following fixation (B). The live cells indicated by the boxed area in the bottom panel of A, are shown in B at high magnification after staining and fixation. The mononucleate produced by cellularisation of a myotube is Sca-1 negative, while an adjacent myoblast is positive.

The same time-lapse approach was applied to investigate Sca-1 staining on mononucleates generated by cellularisation with nocodazole. Here too, all mononucleate progeny were Sca-1 negative. Staining of 401 and nocodazole treated myotubes also showed no expression of Sca-1 on the surface of myotubes (Figure 4-13). These results suggest that the up-regulation of the Sca-1 transcript seen in the microarray experiments may be occurring in the myoblasts.

4.2.3.2 Expression of Sca-2

Sca-2 staining was carried out using a pre-conjugated fluorescein-labelled primary antibody (Pharmingen). Pmi28 mononucleates and purified myotubes on matrigel-coated coverslips were stained as for Sca-1 but no labelling was detected. No induction of the protein was seen in cultures treated with 109 or nocodazole. Despite varying incubation times and antibody dilutions, only occasional, very weak, staining of mononucleates was achieved and was not sufficient to carry out the type of analysis done for Sca-1. Attempts to enhance the signal using the Tyramide Signal Amplification kit (Perkin Elmer) led to high levels of non-specific staining. Unfortunately an un-conjugated form of the antibody was not available as the reagent was recommended for use in cell sorting by FACS. The microarray data suggested that levels of the Sca-2 transcript were present at higher levels than the Sca-1 transcript, both before and after 109 treatment. Despite this information, the Sca-2 protein was not detectable on Pmi28 mononucleates or myotubes given the conditions and reagents used here.

4.3 DISCUSSION

4.3.1 Cellularisation and microtubule-depolymerisation

Microtubules play a vital role in many cellular functions including intracellular transport, signal transduction and cell movement. Perhaps one of the most dramatic examples of the role of microtubules in cellular function is during mitosis, when microtubules form the complex and highly dynamic mitotic spindle. One of the most prominent disruptions of cell activity brought about by agents which disrupt the microtubule network is the blockage of mitosis and because of this, some microtubule agents have been used as anticancer therapeutics. A large range of microtubule-interfering agents have been found to occur naturally in various plants and are highly active on mammalian microtubules.

Colchicine is one of the best characterised of these molecules and was discovered to bind to tubulin and disrupt microtubule dynamics, inhibiting polymerisation.

Nocodazole is a microtubule-depolymerising molecule and binds competitively with colchicine. It forms a complex with soluble tubulin heterodimers and once it becomes incorporated into the polymerising microtubule it inhibits the exchange of tubulin at the microtubule end affecting both incorporation and loss (Hyams and lloyd, 1994). Taxol is another naturally occurring molecule and binds preferentially to polymerised microtubules with little or no affinity for soluble tubulin heterodimers. Its binding site is on the β-tubulin subunit, at the N-terminal and is distinct from that of nocodazole. Taxol is a microtubule-stabilising agent, the microtubule dynamics at both ends of the microtubule are altered, this time favouring the polymerisation of microtubules (Hyams and Lloyd, 1994). The microtubules formed are very stable and at high concentrations of the drug the polymer mass within the cell increases, depleting the concentration of soluble tubulin and inducing microtubule bundling. Dynamic instability and treadmilling are suppressed with both compounds.

The colchicine binding domain is well characterised and consists of two sites, both on the β-tubulin subunit which bind the A and C rings of the molecule (Uppuluri et al., 1993). The B ring interacts with α-tubulin but while the A and C rings are essential for tubulin binding, colchicine analogues lacking the B ring remain active (Mollinedo and Gajate, 2003). Although nocodazole does not share a high level of homology with colchicine, it has been suggested that its structural similarity with the A ring of colchicine enables it to bind at the same site (Jordan and Wilson, 1998).

Another compound, combretastatin A-4, isolated from the bark of a South African tree species, also binds at the same site as colchicine (Mollinedo and Gajate, 2003) and is shown in Figure 4-1. The functional groups on the C ring of colchicine and the B ring of combretastatin A-4 are thought to be the key atoms mediating the interaction with tubulin, while the A ring acts as an anchor and adds strength to the binding (Gaukroger et al., 2003). The two substituted benzene rings of combretastatin A-4 give the molecule a high structural similarity to myoseverin. Structure-activity relationship studies on myoseverin (Perez et al., 2002) revealed an absolute requirement for the two substituted

benzene rings as can be seen by comparing the structure of myoseverin with the inactive control compound YC41 (see Table 3-2). The importance of these two substituted rings is further underlined with the discovery that 109, a triazine-based compound, is capable of the same activity as myoseverin and retains the two hydroxy-benzene groups. The inferred conclusion from studies with 109 and myoseverin, that the purine ring of myoseverin is not important to its activity, is supported by comparison with combretastatin A-4 in which the two rings are joined only by a carbon linker. These observations strongly suggest that 109 and myoseverin are likely to bind at the same site on tubulin as nocodazole.

Nocodazole has been reported to be rapidly reversible (Zieve et al., 1980) and can be used to study the regrowth of microtubules after washing out the drug (Musa et al., 2003; Tassin et al., 1985). The reversible nature of nocodazole was confirmed in my experiments and paralleled the behaviour of 109, with the morphological changes wrought by each compound reversed within 24 hours of removing the compounds. Taxol has been reported to bind reversibly to microtubules (Antin et al., 1981; Caplow et al., 1994) but in comparison to the results here with nocodazole and 109, the contraction of cells and clumping together of nuclei was not appreciably reversed after 24 hours in differentiation medium following the removal of taxol and washing of the cells. The percentage of MHC positive mononucleates remained high also suggesting that fusion was blocked in these cultures. However, this apparent lack of reversibility may be due to the observation that taxol can accumulate within the cell and after just a few hours reach concentrations several hundred fold higher than in the medium (Jordan et al., 1996). This means it can be difficult to wash out taxol and a high concentration may remain in the cells for longer. The effect of taxol on Pmi28 myotubes may have been reversible if the cells had been cultured for several days in fresh medium following drug treatment although the prolonged effect of a microtubule poison such as taxol has implications for cell toxicity.

The fact that nocodazole and taxol bind at different sites on tubulin and that one is a microtubule-binding molecule while the other is a microtubule-stabilising agent makes it difficult to reconcile the observations made here that both compounds are capable of bringing about cellularisation of mammalian myotubes. The process of cellularisation

seems much more consistent with a role for microtubule depolymerisation and might be expected to be blocked by the action of a molecule which stabilises microtubules. The effect of various microtubule-interfering agents can vary according to cell type and stoichiometry of binding. However, at low concentrations of both molecules where there is no appreciable increase or decrease in the overall mass of polymer, the effects of both molecules on microtubule dynamics is the same. At such concentrations where there is no depolymerisation of microtubules, effects brought about by alterations in the microtubule dynamics can be distinguished without confusing them with the effects of depolymerisation (Jordan et al., 1992; Jordan et al., 1993). These experiments reveal that despite their different interactions with microtubules, the two molecules actually affect dynamic instability and treadmilling in a remarkably similar way and highlights the fact that aside from the more obvious effects on the levels of polymerised and soluble tubulin, there are complex but also similar effects on microtubule dynamics corresponding to a decrease in both the rates of lengthening and shortening, increases in the length of time spent in attenuation where the microtubules are neither growing nor shrinking, and changes in the frequency of switching between catastrophe and rescue.

The concentrations used in my experiments are high enough to begin to affect the polymer mass within the cells. Comparison with control cells reveals that there is a decrease in the mass of microtubules in nocodazole treated cells, although there is not complete depolymerisation, while taxol treated cells appear to show an increased intensity of tubulin staining and some evidence in mononucleates of the poorly understood phenomenon of microtubule bundling in response to taxol (Jordan et al., 1993). The effects of the compounds is more extensive in mononucleates than in myotubes. In nocodazole treated mononucleates all microtubules appear shortened and broken with small fragments scattered in the cytoplasm and loss of an overall orientation or organisation. In the myotubes, while the organisation of the microtubules is clearly disrupted, the effects of the two compounds are not so dramatic as in the mononucleate cells. One reason for this may be that the microtubules within myotubes are inherently more stable than in the mononucleates due to post-translationally modifications to the tubulin which accompany myogenesis, these microtubules have been shown to have a greater resistance to depolymerising agents (Chang et al., 2002; Gundersen et al., 1989).

Interactions between the actin and microtubule networks have been observed in a number of vital cellular processes. Proteins able to bind both actin and tubulin can provide physical links between the two systems; additionally they appear to be able to regulate one another via signalling cascades. The interactions between actin and microtubule cytoskeletons are not fully understood but seem to be important in a number of processes which involve changes in cell motility or cell shape, such as axonal guidance, cell migration and wound healing (Rodriguez et al., 2003). Despite communication between the two systems during some morphological changes, cells in which the microtubule cytoskeleton was severely disrupted by 109, retained a largely intact actin network. This was also reported in cells treated with myoseverin. Elongation of myoblasts has been shown to primarily involve microtubules, with little actin at the growing tips (Saitoh et al., 1988) suggesting that during myogenesis, it is the microtubule cytoskeleton specifically which makes a major contribution.

Clearly microtubules have a role to play in maintaining the shape and integrity of myotubes. The nucleation and organisation of the specialised microtubule array is not yet fully understood but the results described here with nocodazole, taxol and 109 suggest that despite an increase in the overall stability of the microtubule cytoskeleton, the dynamic properties of microtubules remain integral to their function and interfering with those properties has consequences for the integrity and morphology of the cell.

4.3.2 Microarray analysis of cellularisation

The discovery of nocodazole as a microtubule-binding molecule with cellularisation activity was in contrast to the original report of myoseverin which claimed other microtubule-depolymerising agents did not induce cellularisation, and added a new dimension to the investigation of the mechanism of action of 109, placing the emphasis firmly on microtubules. This finding directed the design of a microarray experiment to characterise cellularisation, and the role of microtubules in the process, at a molecular level. The array study was designed to expand on the original work carried out with myoseverin by increasing the number of transcripts available for expression analysis and using earlier time points to capture gene expression changes that may be missed by using only a 24 hour reading. The direct comparison of 109 with nocodazole enabled questions to be asked about the mechanism of cellularisation that were not possible in the original

study. In addition to investigating the role of microtubules, the arrays also addressed issues of how far cellularisation represents a reversal in the differentiation of the myotube and whether this phenomenon is of relevance to regeneration research.

All experiments were carried out on the complex, differentiated culture system, containing mononucleates as well as myotubes, previously used for the cellularisation assay. Purification of myotubes has been previously described in materials and methods and has proved a valuable procedure to isolate myotubes and simplify the culture for observation of morphological changes. However, the purification is time-consuming and can give a low yield of myotubes, consecutive purifications can vary in the number of myotubes remaining and in the levels of contaminating mononucleates. Mononucleates remaining after purification tend to divide rapidly in the absence of any contact inhibition causing concern that the activation of transcriptional programs in the surviving mononucleates could mask all but the most extreme changes taking place in the myotubes. The purpose of the microarray study was to look at broad patterns of gene expression and identify marker genes differentially regulated over the course of the experiment. Given that the experiment was aimed at picking up large changes in gene expression, of a magnitude to be detectable even if regulated in only a small population of cells, for example the mononucleates resulting from cellularisation, and was not designed to identify and statistically validate every subtle or transient gene expression change, these mixed differentiated cultures were considered an appropriate and more reproducible system for the microarray experiments.

The goals of the microarray study were reflected in the experimental design, the choice of conditions and the numbers of replicates. For 109 and nocodazole, RNA was extracted at 0, 12 and 24 hour time points in triplicate to enable comparison of the major characteristics of cellularisation by these two molecules. Genes which were just transiently altered or altered to only a small degree in a small number of cells would be likely to be missed, however earlier results from optimisation of my assay to detect cellularisation showed that the percentage of MHC positive mononucleates continued to rise over a 24 hour period so genes specifically regulated in response to cellularisation would perhaps be expected to change over the 24 hour period reaching a maximum at the end of that time. Given the nature of the cultures and the magnitude of changes of

interest, it seemed that I would be most likely to pick up changes in genes which were consistently up- or down-regulated over the course of the experiment making the 24 hour time point arguably the most relevant. As a result of this, treatment with the inactive compound, 401, was carried out for 24 hours as a control for gene expression changes occurring in the culture in differentiation medium over the time course of the experiment.

The application of functional annotation to the lists of genes generated after statistical analysis allowed the genes to be categorised in a manner conducive to the identification of biologically relevant trends and observations. By comparing the frequency of gene categories in the lists with their frequencies on the chip itself I hoped to dissect out the more interesting patterns and identify the association of specific cellular processes with particular treatments. This type of analysis provides a control for any bias in gene categories represented on the chip, or a bias in annotation as genes which are more widely studied are likely to carry more comprehensive annotation. An observation that 50% of all genes isolated in a microarray study are involved in a particular process is of questionable significance if 50% of all genes on the chip are also involved in that same process. The analysis carried out here provides a measure of the probability that a randomly chosen selection of sequences from the chip would carry the same distribution over gene categories. By using a classification system which enables the same gene to fall under multiple categories, this analysis provides some form of control, preventing the investigator from categorising genes based on preconceptions and thus hopefully allowing impartial annotation of genes which may have a role in wound healing under some conditions, but may also be affected during other unrelated cellular process. In this way I have aimed to improve upon the original analysis carried out for myoseverin.

The small number of genes regulated by all three molecules highlights the significant disruption to cellular processes brought about by depolymerisation of microtubules and emphasises the importance of the cytoskeleton in so many aspects of cell behaviour. The largest overlap of genes in any two conditions is that between nocodazole and 109. These two molecules are structurally distinct yet apparently bring about the same effects on multinucleate myotubes, the degree of overlap in the genes involved suggests that they may share a common molecular mechanism. Despite the fact that 109 and nocodazole may act on microtubules in the same way, it is notable that the number of

genes regulated by both 109 and 401, molecules with almost identical structure, is higher than that regulated by nocodazole and 401. This discrepancy can be interpreted in several ways.

The genes that are altered by 109 and 401 but not by nocodazole may constitute a set of transcripts which are altered by effects of 109 brought about independently from its ability to bind and depolymerise microtubules. 109 and 401 are structurally almost identical and it is possible that they may have alternative common targets in the cell resulting in changes to gene expression. Although there is no direct evidence that 109, myoseverin or their derivatives bind to other cellular targets, the absence of data from an untreated culture at 24 hours means it is difficult to entirely discount this possibility. Alternatively, given that nocodazole appears to have more extensive effects on gene expression, as seen by the number of genes regulated, these genes affected by only 109 and 401, may reflect processes which are affected solely in response to nocodazole preventing this set of genes from being up- or down-regulated as they would be in the absence of the drug.

Examination of the types of processes affected by 109 revealed significant regulation of genes involved in forming, or interacting with, the cytoskeleton. This was also seen for nocodazole in agreement with their activity as microtubule depolymerising agents with striking effects on cellular morphology. The fact that cytoskeletal genes were also a feature of the list of transcripts regulated by both molecules gives support to a role for microtubules in the process of cellularisation. The genes in this list were also enriched for muscle related transcripts with up-regulation of genes associated with a myogenic fate. This clearly indicates that dedifferentiation is not occurring at a molecular level in response to 109 or nocodazole and implies that cellularisation is not in itself sufficient to induce dedifferentiation in mammalian myotubes.

The pattern of up-regulation of muscle-associated genes is borne out by examination of the individual lists of genes regulated by 109 or by nocodazole although it is more pronounced in the response to nocodazole, with late markers of muscle differentiation such as myosin heavy chain and acetylcholinesterase up-regulated. It is difficult to interpret these results, there is no evidence or reports that 109 or nocodazole induce

myogenic differentiation. One possibility is simply that the changes in cell shape and volume, and the physical contraction of the myotubes, may require synthesis of these proteins, many of which have a structural role or form part of the contractile machinery.

The core set of 23 genes affected by both 109 and nocodazole is characterised by an up-regulation of injury response genes, more specifically those involved in a defence response, down-regulation of genes involved in signal transduction, negative regulation of growth and up-regulation of muscle-related transcripts. Whether this set of genes is necessary or sufficient for cellularisation is difficult to say. It is clear that 109 and nocodazole do not bring about entirely analogous effects on cell behaviour despite their shared ability to bring about cellularisation of mammalian myotubes, and just as nocodazole will have specific effects on the cell, so will 109. This can lead one to speculate that while nocodazole has wide ranging effects on cell behaviour, a proportion of the genes and processes affected are regulated, in the same way as by 109 and it is this particular subset of genes, among the other processes being affected by the molecule, which brings about cellularisation in the distinct context of a myotube culture.

Another intention of the microarray study was to evaluate some of the hypotheses of the original work with myoseverin in the context of my own system, to look at whether a regeneration-type response was being seen and now by exploiting the properties of nocodazole whether any such response was unique to 109 or a property of any microtubule-binding molecule. Although some of the genes regulated by 109 overlap with those regulated by nocodazole and by 401, over 60% of the genes are significantly altered only in the presence of 109. One question then is the significance of these specific effects and whether they hold any relevance for regeneration.

The original work by Rosania et al proposed that myoseverin activated pathways involved in wound healing and the tissue response to injury (Rosania et al., 2000). The 93 genes regulated by myoseverin included nine growth factors and a number of growth-factor inducible transcripts. Cytoskeletal genes affected were largely down-regulated while genes involved in protein turnover were up-regulated. A number of genes implicated in recruitment of immune cells were up-regulated as were genes with a role in detoxification or the response to oxidative stress. Genes involved in cell cycle

regulation, muscle specific processes, metabolism and macromolecular synthesis were largely unaltered.

In contrast to these results from the microarray with myoseverin, there was no up-regulation of growth factors or genes involved in a growth response with 109; instead there was a clear pattern of negative regulation of the cell cycle and of proliferation, further evidence that cellularisation is not accompanied by dedifferentiation. Of specific interest in the context of which this array study was conducted was the observation that for 109 regulated genes, one of the categories containing an enriched number of sequences was injury response. This category was defined by me based on terms selected from the processes described in the microarray study with myoseverin and those which I felt described a regeneration-like response. Closer examination of the genes in this category suggests that the 109 treated cells are responding to stress but processes which are generally accompanied by cytotoxicity such as inflammation and wound healing are absent or down regulated, while genes involved in detoxification and cellular defence are being activated. The results from my array analysis certainly do not support the scale of the response suggested by the myoseverin work. Rosania et al claim that greater that 50% of the transcripts differentially regulated by myoseverin were involved in the types of processes discussed here, while the genes regulated by 109 and involved in injury response represent only 13% of the total.

Other microarray studies have focussed on profiling the gene expression changes which occur following skeletal muscle injury induced by freezing (Summan et al., 2003), or cardiotoxin (Goetsch et al., 2003), or in the skeletal muscle of mdx mice which undergoes cycles of degeneration and regeneration (Porter et al., 2002). In all cases the early response, within 24 hours, is dominated by inflammation and an up-regulation of chemokines and chemokine receptors followed by induction of cell cycle and proliferation genes as satellite cells are activated. If these studies can be said to characterise the physiological response to injury, it is clear that these key aspects are absent from the response to 109. The only chemokine regulated in response to 109 is fractalkine which is down-regulated, the growth response also appears to be negatively regulated.

Interestingly, one microarray study profiling the myogenic differentiation of C2C12 myoblasts (Moran et al., 2002) also reported an up-regulation of some genes involved in modulating the inflammatory response, including transcripts involved in recruitment of immune cells. This perhaps suggests that genes and processes associated with regeneration are also involved in differentiation. One conclusion made in a study attempting to resolve vastly different microarray findings from similar experiments by independent groups, was that differences are more likely to arise when dealing with relatively small fold changes in genes which are already expressed in the cell types under study, rather than with genes which are expressed exclusively in response to a particular treatment or in a given cell type (Fortunel et al., 2003). The work referred to in that study involved stem cells and highlights some of the problems and discrepancies that arise when different experimenters carry out microarray research. A number of studies have sought to characterise a genetic fingerprint for the elusive property referred to as 'stemness' by comparing the expression profiles from different types of stem cell with the aim of isolating a core set of genes that confer the common functions of stem cells such as self-renewal and pluripotency. Two independent groups claimed to have each isolated such a set of genes using microarrays (Ivanova et al., 2002; Ramalho-Santos et al., 2002), but a third group carrying out their own study analysed their results alongside those of the earlier studies and found only one gene in common between the three (Fortunel et al., 2003).

The stem cell studies emphasise the importance of cross-referencing independently derived data from different investigators and warn of the potential problems of using microarray technology to definitively characterise a complex process. It is unlikely that different microarray experiments will yield an identical list of genes but despite some experimental differences, robust trends in the gene expression data for myoseverin would be expected to persist in my analysis if it were to be concluded that these small microtubule-binding molecules are indeed activating a programme in mammalian cells indicative of that occurring during urodele regeneration. This concept is demonstrated in the case of three independent microarray studies on the myogenic differentiation of C2C12 myoblasts (Moran et al., 2002; Shen et al., 2003; Tomczak et al., 2004). While each study has a slightly different focus and identifies different genes, the characteristic motifs of the process, such as down-regulation of cell cycle and DNA replication genes

with concomitant up-regulation of adhesion or fusion-related transcripts, components of the muscle contractile machinery and cell cycle inhibitors, persist in all three cases. My microarray results, with Pmi28 myotubes and compound 109, do not support induction of the type of regeneration-like programme described by Rosania et al, in mammalian cells at a molecular level.

In addition to using the microarray analysis as a tool to study the role of microtubules in cellularisation and investigate the relevance of changes in gene expression in the context of regeneration, a secondary consideration was the identification of genes that could be used as markers of the cellularisation process. The application of markers may enable identification of a specific cell population following cellularisation, provide a quantifiable index of changes taking place within the culture, or further characterise events at a single cell level. Following cellularisation, the culture contains a population of cells that did not exist before, i.e. the mononucleates generated by fragmentation of the myotubes, it was hoped that the discovery of marker genes may help to characterise these cells and determine whether they represented a subpopulation in the culture with unique properties. Other markers may also illuminate any changes occurring specifically in myotubes. Ideally the chosen markers would be detectable at the protein level by immunohistochemistry.

Sca-1 and Sca-2 were chosen as appealing candidates. The cell surface localisation of the antigens and availability of commercial antibodies provided a reasonable chance that the protein could be detected by standard immunocytochemical techniques. Although Sca-1 was up-regulated by the control compound as well as by 109 and nocodazole, its role in stem cell biology nevertheless made it an interesting target given the issues of dedifferentiation surrounding cellularisation. In conjunction with a second stem cell marker, Sca-2, which was regulated specifically by 109 and nocodazole, identification of the cell types expressing these genes, posed an interesting challenge.

Sca-1 and Sca-2 are both members of the Ly6 family of cell surface antigens, their exact cellular functions are not fully understood and ligands of both molecules are poorly characterised. Sca-1 is a marker for haematopoietic stem cells (HSCs) but is down-regulated during thymocyte development and re-expressed on most mature peripheral

lymphocytes and thymocytes. The Sca-1 null mouse is viable and all haematopoietic lineages are present (Ito et al., 2003; Stanford et al., 1997) showing that Sca-1 is not required for development. The HSCs, however, show impaired self-renewal and ability to repopulate a lethally irradiated bone marrow compartment although Sca-1 does not appear necessary for HSC maintenance under normal conditions (Ito et al., 2003). Sca-1 expression is associated with activated T cells, although conversely Sca-1 null lymphocytes are reportedly hyperproliferative in response to activation through the T cell receptor (Stanford et al., 1997), suggesting that Sca-1 may have a complex regulatory role in T cell activation. Sca-2 is not expressed on HSCs but is coexpressed with Sca-1 on early thymic precursors accompanying committment to the lymphoid lineage, which suggests an important role in T cell development (MacNeil et al., 1993). In addition to expression on thymic precursors, Sca-2 is found on mature B cells and some non-erythroid bone marrow populations (Antica et al., 1997). Expression of both Sca-1 and Sca-2 has been detected on non-lymphoid tissues including liver, brain and heart suggesting their function is unlikely to be lymphocyte specific (Gumley et al., 1995).

The issues surrounding the identification of suitable markers highlight one of the disadvantages of using a mixed population of cells for microarray studies. Although, this approach was chosen to provide a reproducible system and contains all cell types of a differentiated culture, the resulting microarray data gives no indication of whether genes are regulated within myotubes, mononucleates, or by a subpopulation of either of these cell types. Differential regulation by the same gene in different cell types will also be masked.

Sca-1 was readily detected on Pmi28 mononucleates using a monoclonal antibody and the staining pattern was strongly indicative of a cell surface protein; loss of staining after treatment with PIPLC confirmed that Sca-1 is GPI-anchored. GPI-anchored proteins are thought to associate in lipid rafts on the membrane where they act as coreceptors, many have been found to interact with protein tyrosine kinases suggesting a role for these proteins in cell signalling (Loertscher and Lavery, 2002; Simons and Toomre, 2000). Immunohistochemistry revealed no detectable expression of Sca-1 on myotubes either before or after treatment with any of the three compounds while almost all myoblasts expressed the protein to varying degrees. These results suggest that the gene is regulated

within mononucleates and is independent of the effects of 109 and nocodazole on the cytoskeleton. Along with the negative correlation between Sca-1 and MHC expression in mononucleates, the observed staining patterns infer that Sca-1 is expressed on the surface of proliferating myoblasts and is lost as the cells undergo differentiation, the differences in expression levels among cells may represent intermediate stages of differentiation, or may define subpopulations with a range of myogenic potentials.

The reason for up-regulation of Sca-1 in mononucleates is unknown. The functions of the protein are not fully understood. No specific role in myogenic cells has been identified although Sca-1 positive cells have been isolated from skeletal muscle (Asakura et al., 2002; Gussoni et al., 1999; McKinney-Freeman et al., 2002). These cells have been referred to as muscle-derived stem cells and are distinct from satellite cells. They have been shown to possess the ability to differentiate into cells of the haematopoietic lineage and, under conditions of muscle regeneration, may give rise to satellite cells (Asakura et al., 2002) although their exact origin and relationship to satellite cells remains unclear. Three independent microarray studies characterising the differentiation of C2C12 cells in culture all reported a peak in Sca-1 expression corresponding approximately to the onset of differentiation (Moran et al., 2002; Shen et al., 2003; Tomczak et al., 2004) although the significance of this observation in terms of myogenesis is unknown. In the context of my results, this may suggest that the regulation of Sca-1 in the Pmi28 cultures indicates ongoing commitment to differentiation, even in the presence of microtubule-binding molecules, and that mononucleates expressing high levels of Sca-1 are initiating a programme of terminal differentiation. Some studies have implicated Sca-1 in cell-cell adhesion (Bamezai and Rock, 1995; English et al., 2000), although a specific role in myoblast recognition or fusion has not been reported.

In contrast, Sca-2 was undetectable on either mononucleates or myotubes using a fluorescein-conjugated monoclonal antibody, and was not induced in either population in response to 109 or nocodazole despite the microarray results, which indicated that the Sca-2 transcript was expressed at much higher levels relative to Sca-1. One inherent problem in using microarray data in this way is that changes in the levels of mRNA do not necessarily transform directly into changes in the amount of the corresponding protein. Stability of the message, regulation of translation, and post-translational

modification of the protein itself add multiple levels of complexity to the process. The differences in expression levels of the Sca-1 and Sca-2 proteins may be due to an increased rate of turnover of the Sca-2 protein or to differing proportions of intracellular and plasma membrane-bound molecules, the staining protocol used for these experiments dictates that only antigen present at the cell surface is detected. Sca-2 is less widely studied than Sca-1 and little is known about its function, its expression in skeletal muscle has not been previosly reported.

Another possibility for this discrepancy is that manipulations during the course of the experiment may have altered the expression of cell surface molecules. The microarray study was carried out on dense mixed differentiated cultures plated on collagen I-coated plastic dishes. In order to examine the staining of membrane proteins more carefully the cells were purified and plated onto matrigel-coated glass coverslips. Matrigel is a soluble extract of basement membrane, rich in extracellular matrix components. Cell-cell and cell-matrix interactions can influence the types and amounts of molecules expressed on the cell surface and both of these interfaces have been disrupted during purification.

Initial Sca-1 staining of dense myotube cultures on silane-coated glass coverslips suggested that myotubes were Sca-1 negative but at high magnification the high density of the culture made it difficult to distinguish the membrane of one cell from that of its neighbours. While this supports the proposal that purification did not alter the expression pattern of sca-1 it cannot completely rule out the possibility that regulation of the gene may be altered by the change in cellular environment. The problem remains that the conditions used for the microarray experiments are not favourable for the detection of Sca-1. The Sca-1 staining is relatively weak and is only detectable at high magnification, when cells are stained on plastic culture dishes, an increased signal to noise ratio effectively decreases the intensity of the signal. The use of glass in place of plastic often offers improved optics for immunofluorescence, but, conversely, purified myotubes do not adhere well to a glass surface, which necessitates the use of additional matrix materials.

Despite the fact that neither Sca-1 nor Sca-2 can be used to identify the newly formed mononucleates resulting from cellularisation, Sca-1 has nonetheless provided me with a

potentially useful marker for future studies. Investigation of the consequences of cellularisation on the behaviour and differentiated state of these mononucleates will be aided by the use of markers for various stages of differentiation. Although the mononucleates generated by fragmentation of myotubes initially appear to be Sca-1 negative, like their parent myotubes, acquisition of a Sca-1 positive phenotype can potentially be used as a marker for a certain degree of reversal in the differentiation process in these cells over time, or in response to further manipulations.

4.3.3 Conclusions

The results of this chapter have highlighted the role of microtubules in cellularisation of mammalian myotubes. Earlier time-lapse microscopy work revealed extensive changes to cellular morphology in response to treatment with 109, indicative of significant cytoskeletal reorganisation. The findings that nocodazole was also capable of inducing cellularisation of Pmi28 myotubes supported the hypothesis that 109 brings about cellularisation through its effects on microtubules and lent a novel aspect to the subsequent microarray study. While the importance of microtubules in maintaining the unique morphology of myotubes is clear from this work, the actual mechanism by which the disruption of microtubule dynamics ultimately leads to narrowing at points along the length of the myotube and eventually fission at these points to generate viable fragments, remains obscure. The fragmentation of myotubes into 'myosacs' in response to microtubule-depolymerising agent has been previously documented, by a mechanism resembling the action of 109, at least at the level of morphological description (Bischoff and Holtzer, 1968; Saitoh et al., 1988), although formation of viable mononucleate progeny was not reported.

Although there is no indication of dedifferentiation on a molecular level, cellularisation itself can be said to represent a reversal of one aspect of myogenic differentiation by generating mononucleate cells from a terminally differentiated myotube which is, by definition multinucleate. Even though I suggest there is no regeneration response on the scale suggested in the myoseverin paper, immune response genes were enriched with 109 while not with the other compounds and observations such as the down-regulation of adhesion molecules in response to 109 pose interesting questions about the fate of those newly formed cells.

It seems unlikely that these specific gene expression changes, unique to treatment with 109, are an integral part of the cellularisation process given that cellularisation itself is not a process unique to 109. Indeed, the inclusion of nocodazole in the microarray study has provided a valuable tool for dissection of the process of cellularisation and allows a new perspective on the subject of regeneration and cellularisation. However, while cellularisation can happen simply as a response to microtubule depolymerisation, the consequences of reversing the mononucleate to multinucleate transition, for the individual mononucleate progeny, remains to be determined and raises a number of questions; can the specific molecule responsible for cellularisation determine the consequences of the event? Are the mononucleate cells viable? And is cellularisation sufficient to trigger other aspects of dedifferentiation such as cell cycle re-entry?

Chapter 5

The consequences of cellularisation for the mononucleate progeny of mammalian myotubes

5.1 INTRODUCTION

Previous work using a cellularisation assay and time-lapse microscopy to observe the response of individual myotubes to 109, has clearly established the formation of mononucleate progeny by fragmentation of multinucleate myotubes. Closer examination of the cytoskeleton during cellularisation and the discovery that nocodazole is also capable of the phenomenon highlighted the role of microtubules in this process and hence their apparent role in maintaining the multinucleate aspect of myogenic differentiation. While microarray technology did not support the concept of dedifferentiation during cellularisation at a molecular level, it highlighted some interesting patterns, which could possibly have consequences for the fate of the newly formed mononucleates. The effects of 109 are reversible; the decrease in the number of MHC positive mononucleates in a myotube culture after a period in differentiation medium, following cellularisation, suggests that the mononucleate progeny are competent to fuse with other existing myotubes or mononucleates and represent viable, myogenic cells. However, there has, as yet, been no direct convincing information on the fate and potential of this population of mononucleates.

Despite the fact that there is no apparent evidence for dedifferentiation at a molecular level during the 24 hour period of cellularisation, it should not be forgotten that the multinucleate state is one of the defining features of a terminally differentiated mammalian muscle culture and 109 has in effect reversed this aspect of differentiation. That this action may subsequently have important consequences for the fate of the mononucleates produced is a significant possibility and requires further investigation. While the original work with myoseverin is also conservative in its claims of dedifferentiation, the authors suggest that merely restoring the cells to a mononucleate state may be sufficient to release the cell cycle arrest, also a characteristic feature of terminal differentiation, and allow the cells to divide.

Conditional expression of Msx-1 in terminally differentiated mammalian myotubes has been shown to induce cellularisation of the myotubes, reportedly giving rise to mononucleates which are not only capable of cell cycle re-entry and division but also transdifferentiation, indicative of a reversal in lineage committment. In light of these results and the lack of information specific to 109, I have looked more closely at the fate

of these mononucleates resulting from cellularisation of mammalian myotubes by 109 and attempt to characterise these cells in terms of their viability, proliferative capacity, differentiation state and potentiality.

5.2 RESULTS

5.2.1 Monitoring cell division of mononucleate progeny

5.2.1.1 Following the fate of mononucleates using grid squares

Pmi28 differentiated myotubes were purified and plated onto collagen-coated 35mm dishes scored with a 10x10 grid of approximately 1mm squares. Purified cells were plated at low density to promote the distribution of isolated myotubes within the physical boundary of a single scored grid square. 24 hours after plating, squares containing suitably isolated myotubes were photographed in phase contrast, the dish was then treated for a further 24 hours with 15μM 109. The cells were washed gently with D-PBS to remove traces of 109 and the same grid squares chosen at the start of the experiment were photographed again to look for cellularisation of myotubes. Cells were then maintained in Pmi28 myoblast growth medium for a further four days, grid squares in which a cellularisation event had occurred in response to 109 were photographed at 24 hour intervals throughout this period to catalogue the behaviour of the mononucleates and observe any cases of cell division. At the end of the experiment, cells were fixed and the nuclei stained to confirm the mononucleate nature of the cells of interest. Figure 5-1 summarises the response of 415 myotubes to the initial 24 hour period of treatment with 109. The response was determined by comparison of the images taken immediately before and after the 109 treatment period. 219 myotubes, just over half the total number studied, failed to fragment. In the majority of these cases the myotubes gradually contracted from a long straight myotube with nuclei arranged along the length, to a more compact and rounded cell with nuclei grouped in the centre. A further 164 myotubes underwent some form of fragmentation event resulting in the formation of smaller myotubes and/or mononucleates, from a single myotube. Of the 164 myotubes, 102 myotubes, almost a quarter of the total number followed, gave rise to mononucleate progeny and can therefore be truly classed as cellularisation events. A small percentage of myotubes, less than 8%, contracted rapidly and died.

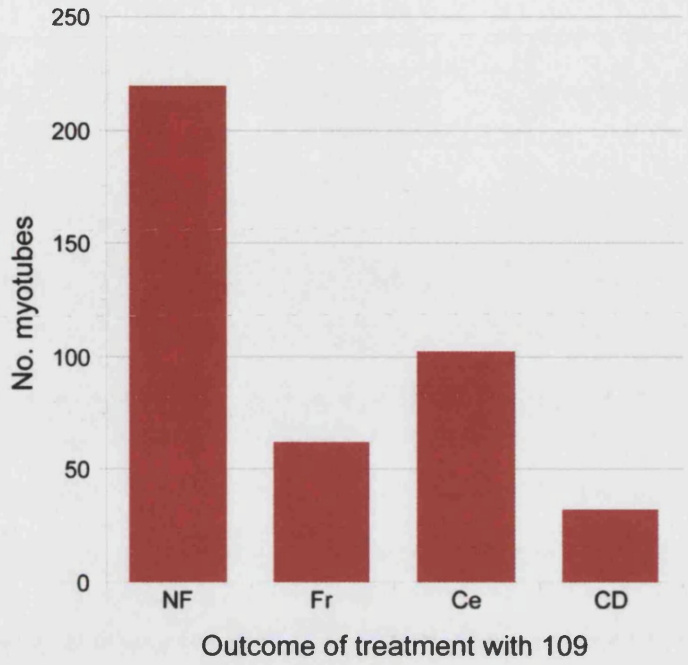


Figure 5-1: The outcome for Pmi28 myotubes of exposure to 109. Pmi28 myotubes were purified and plated at low density onto dishes scored with a grid. Grid squares containing only isolated myotubes, with no contaminating mononucleates, were photographed before and after a 24 hour treatment with 15μM 109. A total of 415 myotubes were analysed, each myotube underwent one of four possible outcomes of the treatment; 52.8% showed no fragmentation (NF), 14.9% fragmented, giving rise to two or more multinucleate cells (Fr), 24.6% underwent a cellularisation event producing a mononucleate cell (Ce), and 7.7% underwent cell death in response to 109 treatment.

Of the 102 mononucleates produced by 109 treatment, 59 of these (in 47 grid squares, over 14 independent experiments) were washed and incubated in growth medium for the following 72-96 hours as described above. The behaviour of the cell of interest was captured at 24 hour intervals. However a number of issues made interpretation of these images difficult. It became clear that the scored boundaries of the grid squares were not forming a sufficient physical barrier to the movement of mononucleates. Even following purification and low density plating of the myotubes, some mononucleates will persist, and their presence in this type of experiment is clearly a complication. A further concern was the discovery, after fixation at the end of the experiment, of contaminating mononucleates at the very edge of the grid squares which had remained undetectable by the low magnification phase contrast microscopy used to image the individual grid squares over the course of the experiment. Pmi28 cells transfected with a nuclear localised eGFP protein, (as described in Chapter 3) were used in place of their untransfected counterparts to try and enable easier detection of contaminating mononucleates at the start, and follow the cells more carefully throughout the

experiment. However, due to transfection efficiency, only 50% of the mononucleates would carry the eGFP label, and concern over the effects of repeated exposure to ultra violet radiation on cell behaviour and viability made this an inadequate solution to the problem. In addition, a key limitation of this approach was that the movement of cells in some of the grid squares over the course of a 24 hour period made positive identification of the same cell in subsequent pictures very difficult.

Despite the inherent problems with this experimental approach, some conclusions can be made albeit with consideration of the caveats described above. The cellularisation events observed in each of the 47 grid squares, giving rise to the 59 mononucleates, were further categorised in terms of whether an increase in the number of mononucleates was observed in that square over the course of 3-4 days in growth medium. In 54% of cases, no increase was seen and in these squares it was usually possible to identify the mononucleate progeny at each 24 hour time point, the morphology and positioning of these cells tended to remain relatively constant making it unlikely that the cells had divided. In the remaining 46% of grid squares, an increase in the number of mononucleates was seen over the course of the experiment. However, in almost two-thirds of these cases, the increase was considered unlikely to be due to division of the mononucleate being followed as either the rate of increase in mononucleates was incompatible with the length of the cell cycle in Pmi28 cells, or the original mononucleate remained identifiable throughout. This leaves 17% of the grid squares in which the increase could reasonably be attributed to division of the mononucleate of interest but the information in the available images was insufficient to distinguish this possibility from that of contaminating mononucleates.

5.2.1.2 Analysis of the proliferative potential of mononucleates by incorporation of BrdU

Bromodeoxyuridine (BrdU) is a base analogue, which can be added to culture medium at micromolar concentrations and becomes incorporated into DNA as the cell enters S phase. The presence of BrdU in the nucleus can then be detected with a monoclonal antibody. In order to characterise the mononucleates created by 109-induced cellularisation I tested the cells for incorporation of BrdU. Pmi28 myotubes were purified and plated onto collagen-coated 35mm dishes then treated with 15μM 109 for 24

hours. BrdU was added to each dish for a 24 hour period, either during 109 treatment or immediately after, when cells were washed to remove traces of 109 and then stimulated with growth medium (containing 20% fetal calf serum) for the next 24 hours. Cells were fixed and stained with antibodies to BrdU and MHC.

As for the cellularisation assay, the persistence of MHC within mononucleates formed by myotube fission was used to identify these cells. As a background level of MHC positive mononucleates is present in all cultures, a control dish was prepared in parallel alongside every dish of myotubes treated with 109. For all dishes an equal area in the centre of each dish, approximately 2.5cm square, was marked prior to plating of the cells and all MHC positive mononucleates in this area were counted and scored as positive or negative for BrdU. Table 5-1 shows the level of incorporation of BrdU into MHC positive mononucleates. Whether BrdU was added along with 109 or during the 24 hours following, the percentage of MHC positive mononucleates that re-enter S phase is not significantly different between 109 treated and control cultures, in all experiments MHC negative mononucleates efficiently incorporated BrdU to high levels.

Table 5-1: Detection of S phase re-entry in MHC positive mononucleates following treatment with 109.

	CONTROL			109 TREATED		
	MHC+ve		MHC-ve	MHC+ve		MHC-ve
Expt	No. cells	% BrdU +ve	% BrdU +ve	No. cells	% BrdU +ve	% BrdU +ve
1	8/136	**5.88**	98	10/248	**0.40**	94
2	0/169	**0**	99	20/376	**0.53**	84
3	2/159	**1.26**	99	0/154	**0**	70
4	3/94	**3.19**	98	3/207	**1.45**	99

1-4 are four experiments, each comprising a pair of dishes, one treated with 109 and one with medium alone. In experiments 1-3, purified myotubes were treated with 15μM109 (or medium alone) for 24 hours then washed in D-PBS and maintained for a further 24 hours in growth medium containing 10μm BrdU. Cells were then washed, fixed and stained with an antibody to detect the incorporation of BrdU. All MHC positive mononucleates in a pre-defined area were scored as positive or negative for BrdU incorporation. In experiment 4, cells were incubated with 10μm BrdU during 109 treatment, then fixed and stained immediately after as for experiment 1-3. In each dish 100 MHC negative mononucleates were also scored for BrdU incorporation as a positive control. The average number of MHC positive mononucleates is increased in 109-treated dishes consistent with cellularisation having taken place, but incorporation of BrdU remains low suggesting the cells formed by cellularisation of myotubes do not re-enter S phase, either during 109 treatment or in response to serum stimulation following treatment.

In a separate series of experiments, S phase re-entry within Pmi28 myotubes in response to 109 was investigated. The onset of myogenic differentiation is characterised by irreversible withdrawal from the cell cycle, the nuclei in mammalian myotubes are

subsequently unresponsive to all mitogens, but in cultured newt myotubes S phase re-entry can be stimulated by the addition of serum. The results with 109 treatment of Pmi28 myotubes (Table 5-2) showed no increase in BrdU incorporation either during or after treatment with the compound.

Table 5-2: Detection of S phase re-entry in myotubes following 109 treatment.

Expt	CONTROL			109 TREATED		
	No. myotubes	Average no. nuclei/cell	% nuclei BrdU+ve	No. myotubes	Average no. nuclei/cell	% nuclei BrdU+ve
1	100	7.48	**0.13**	100	8.30	**0**
2	100	10.52	**0.19**	100	7.46	**0.13**
3	150	14.12	**1.20**	150	16.64	**0.60**

1-3 are three independent experiments, each comprising a pair of dishes, one treated with 109 and one with medium alone. In experiments 1-2, purified Pmi28 myotubes were treated with 15μM 109 for 24 hours, followed by maintenance in growth medium containing 10μm BrdU for a further 24 hours. Cells were then fixed and stained with an antibody to detect incorporation of BrdU. In experiment 3, BrdU was added during the 24 hour 109 treatment, cells were fixed immediately after and stained as for experiments 1 and 2. Only myotubes containing three or more nuclei were included, all nuclei in the myotubes counted were scored as positive or negative for BrdU incorporation. 109 does not induce S phase re-entry in mammalian myotubes either during treatment of in response to serum stimulation following treatment.

5.2.1.3 Determination of cell fate by time-lapse microscopy

As discussed previously, following the mononucleates generated by cellularisation of myotubes by imaging at 24 hour periods was problematic for several reasons, one of which was that the length of time between images was too great to accurately document the movement and behaviour of the cells under study. Only time-lapse microscopy appeared to offer the resolution and coverage necessary to monitor individual cells in detail over time. Use of a motorised stage, with movement in x, y and z planes enabled a high-throughput approach to time-lapse with the successive capture of images from up to twenty pre-determined fields at each chosen time point, and the assembly of twenty near-simultaneous movies from just a single dish of cells.

Purified Pmi28 myotubes were used for all time-lapse microscopy experiments described in this chapter in order to allow clear visualisation of individual cells. A 35mm dish of purified myotubes was placed inside the climate-controlled and pre-equilibrated time-lapse chamber (see Materials and Methods for details), from this point all further experimental manipulations were carried out within the environment of the chamber. During a 24 hour period of treatment with 15μM 109 in differentiation medium, one

image was taken at four or five minute intervals, at each position on the dish (defined by an x, y and z coordinate), in order to capture cellularisation events. Following 109 treatment the dish was washed gently with PBS and the medium replaced with fresh growth medium. For the next 4 days, images at the same coordinates observed during cellularisation, were captured at 30 minute intervals; medium was changed after 48 hours. At the end of the time-lapse period the cells were fixed and stained for a range of antigens (described later) and with a nuclear stain to confirm that cells arising from cellularisation of myotubes with 109, and then followed for the course of the experiment, were indeed mononucleate.

From 13 independent time-lapse experiments, observation of movies from a total of 115 fields, yielded 64 fields in which mononucleates were generated from multinucleate myotubes during the 24 hour treatment with 109. Figure 5-2 shows the fate of 112 mononucleates during four days in growth medium (and 34 during two days in growth medium), in those 64 fields.

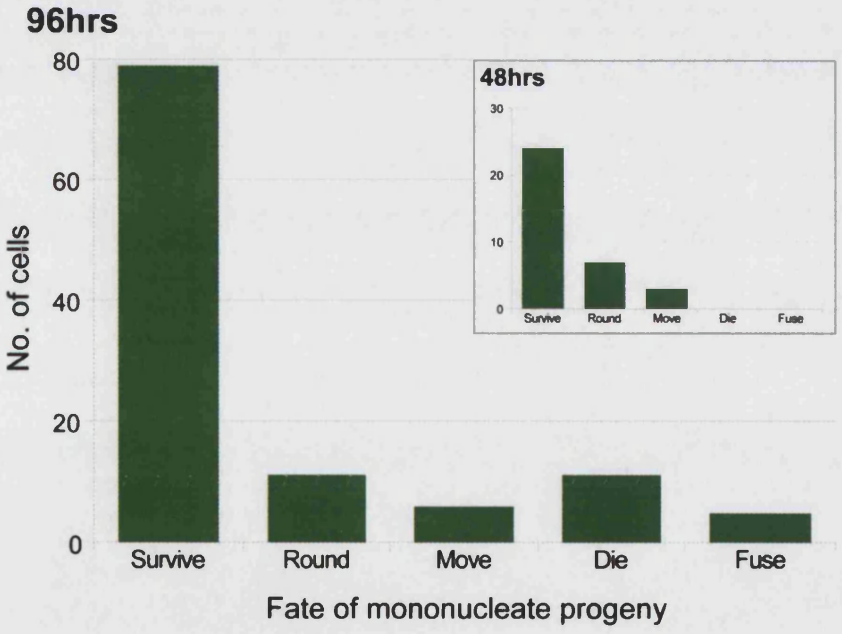


Figure 5-2: The fate of mononucleate progeny arising by 109-induced cellularisation of Pmi28 myotubes. Purified myotubes were treated with 15μM 109 for 24 hours and cellularisation recorded by time-lapse microscopy. Cells were then washed with D-PBS and maintained in growth medium. Mononucleate progeny generated as a result of a cellularisation event were observed by time-lapse microscopy over a subsequent 48 hour (see inset) or 96 hour (main graph) period. A total of 146 mononucleates were followed in this way (112 for 96 hours, 34 for 48 hours). The behaviour of each cell, over the course of the time period followed, could be described by one of five possible categories, each cell either, survived as an elongated, non-proliferating mononucleate (Survive), rounded up but remained attached (Round), moved out of frame and could no longer be followed (Move), underwent cell death (Die), or fused back into an existing myotube (Fuse). The majority of mononucleates remained viable and adherent but showed an elongated morphology; no cases of cell division were observed.

The majority of these mononucleates elongated during the first few hours in growth medium following removal of 109 and then maintained a relatively constant shape, size and position over the subsequent days in growth medium. Figures 3-3A and 3-3B show stills from a time-lapse movie generated as described above and demonstrate a typical example. The time-lapse sequences from which the images were taken are included in Supporting Data as video4A and video4B. Figure 5-3A (video4A) shows cellularisation, with 109, of a myotube over 24 hours resulting in generation of a single mononucleate. The fate of the mononucleate over the next four days is depicted in Figure 5-3B (video4B), the mononucleate under study is indicated with an arrow in each frame and the images are taken from the same field, which demonstrates the limited motility of the cell. The cell survives as a mononucleate for the duration of the experiment showing little change to its morphology.

A smaller proportion of cells remained as mononucleates attached to the dish but rounded up. Some of these cells elongated initially but then contracted after a couple of days in growth medium. A few cells moved out of frame before the end of the experiment and so were excluded from any further analysis. In a very limited number of cases the mononucleate being followed fused with an existing myotube. The remaining 11 cells (7.5% of the total) died and lifted away from the substrate.

None of the 146 mononucleates generated by 109-induced cellularisation of myotubes were observed to undergo cell division over the course of the experiment. In contrast, background myoblasts were seen to undergo multiple cell division cycles in growth medium following removal of 109, providing evidence that the culture conditions were appropriate to support proliferation. No cell division was seen during 109 treatment, consistent with the depolymerisation of microtubules that are required for formation of a mitotic spindle.

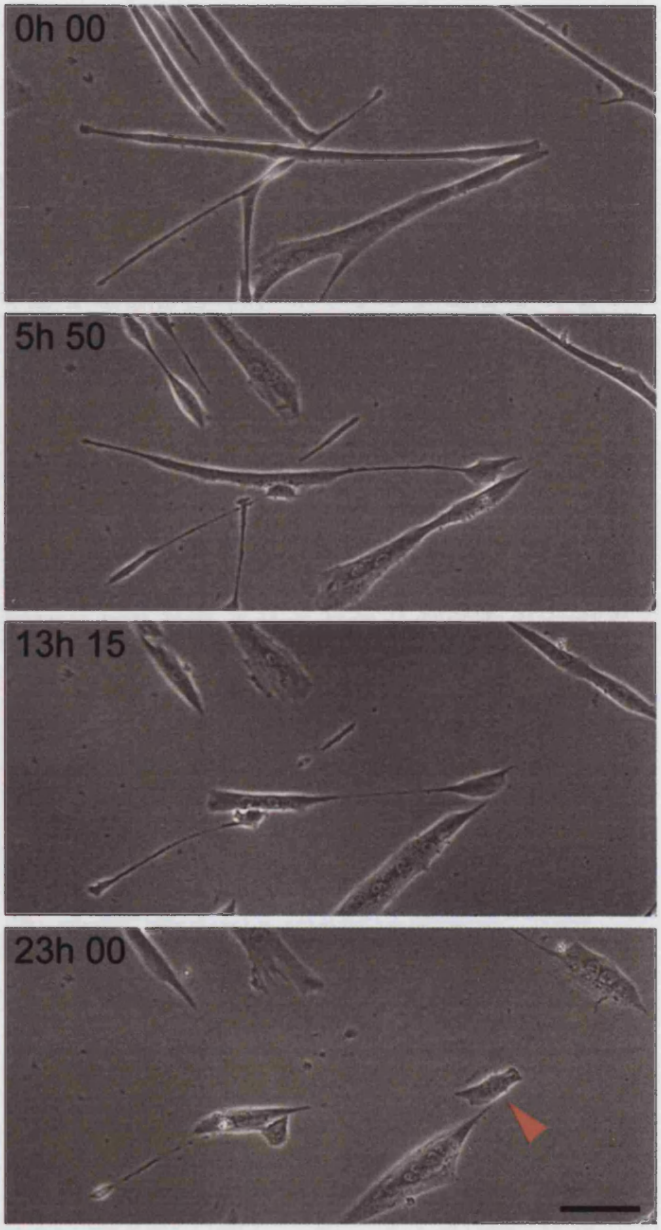


Figure 5-3A: Cellularisation of Pmi28 myotubes to enable study of the mononucleate progeny by time-lapse microscopy. Purified myotubes were treated with 15µM 109 for 24 hours, then washed in D-PBS and maintained in growth medium for a further 96 hours. Chosen fields were imaged throughout by time-lapse microscopy to follow the behaviour of mononucleate cells arising by cellularisation. Selected stills from a time-lapse movie are shown here to illustrate the cellularisation of an individual myotube giving rise to a single mononucleate, indicated by an arrowhead; the subsequent behaviour of the marked cell is shown in Figure 5-3B. Scale bar = 100µm.

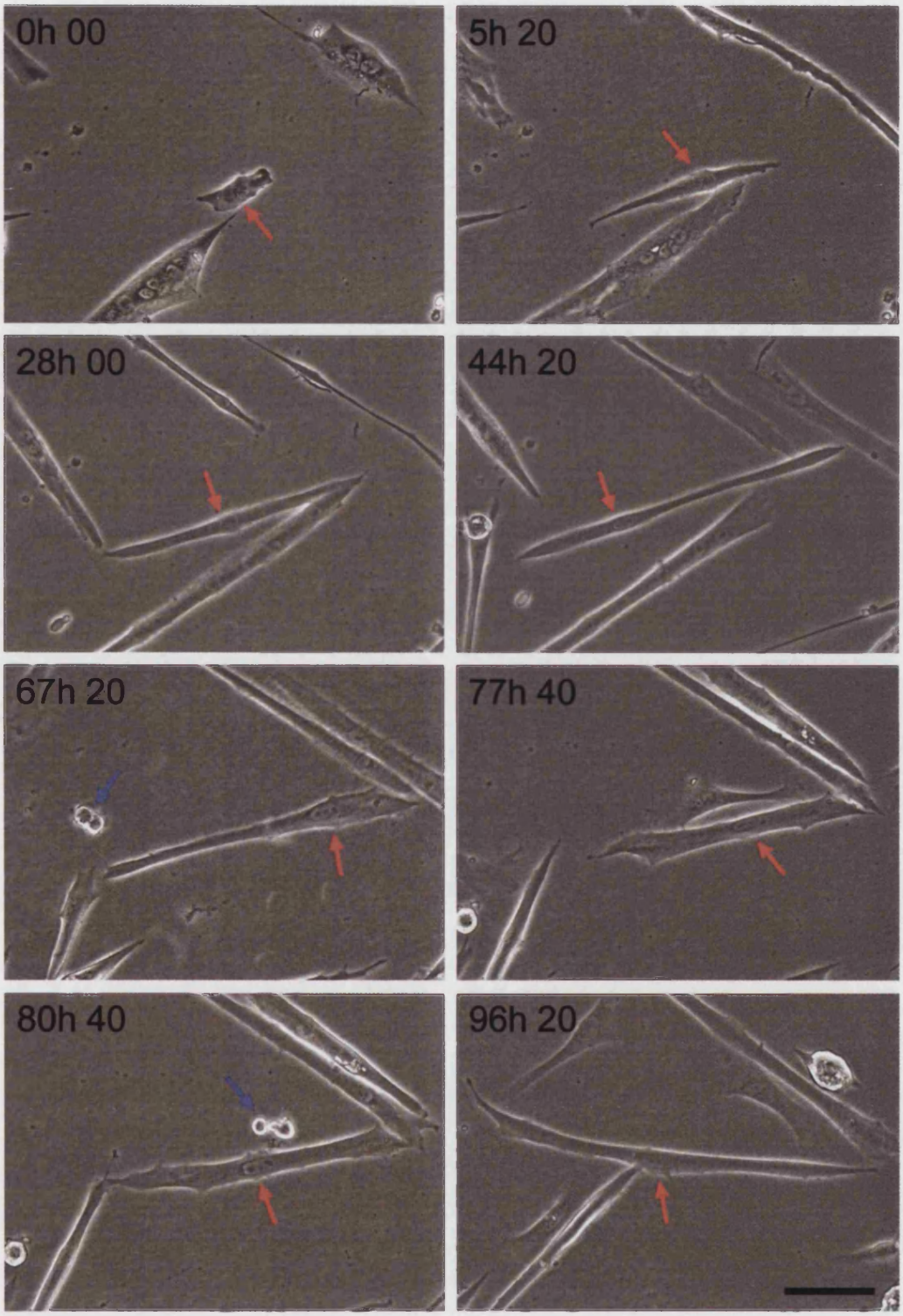


Figure 5-3B: Following mononucleate progeny of cellularisation by time-lapse microscopy. Purified myotubes were treated with 15μM 109 for 24 hours, then washed in D-PBS and maintained in growth medium (GM) for a further 96 hours. Chosen fields were imaged throughout by time-lapse microscopy to follow the behaviour of mononucleate cells arising by cellularisation. Selected stills from a time-lapse movie are shown here to illustrate the typical morphology of mononucleate progeny over the 96 hours in GM following cellularisation, the cellularisation event is shown in Figure 5-3A. The mononucleate cell elongates but then maintains its shape and position demonstrating very little motility, the same cell is marked with a red arrow in each image. Dividing myoblasts in the same frame are marked with blue arrows, indicating that culture conditions are sufficient for proliferation. Scale bar = 100μm.

5.2.2 Staining of mononucleate progeny for differentiation markers

5.2.2.1 Expression of MHC by mononucleate progeny

As mentioned above, cells followed by time-lapse microscopy were fixed and stained at the conclusion of the experiment to further characterise the mononucleates arising as a result of cellularisation events, in terms of expression of a range of specific proteins. Given that the cells did not appear to undergo division, some dishes were stained for MHC to determine whether the mononucleates under study were still expressing markers of terminally differentiated muscle.

Of 21 mononucleates stained for MHC after four days in growth medium, all still expressed levels of MHC comparable with those seen in myotubes on the same dish. Figure 5-4 demonstrates the cellularisation of a single myotube, the subsequent behaviour of the resulting mononucleates and finally their fixation and staining for MHC. These images are stills from video5A and video5B, included in Supporting Data. Although the cells are close together at some stages, the individual mononucleates can be followed throughout by careful observation of the movies and are marked by arrows in Figure 5-4. After four days in growth medium, MHC was clearly detected in these mononucleates. In some cases, striations in the pattern of MHC staining were seen in mononucleates resulting from cellularisation and followed for four days. Such striations are sometimes seen in myotubes and are indicative of late differentiation and organisation of the muscle contractile machinery. This data suggests that the mononucleates are not undergoing dedifferentiation.

Myoseverin treated Pmi28 myotubes were also followed by time-lapse microscopy to avoid the suggestion that the differences in structure between myoseverin and 109 may be responsible for differences in their ability to generate dividing mononucleates. In two independent experiments, 35 mononucleates in 18 separate fields were followed. 23 of these cells survived as mononucleates and did not move out of field. After four days in growth medium none of these cells had undergone division and staining of the cells for MHC revealed that all had retained the differentiation marker.

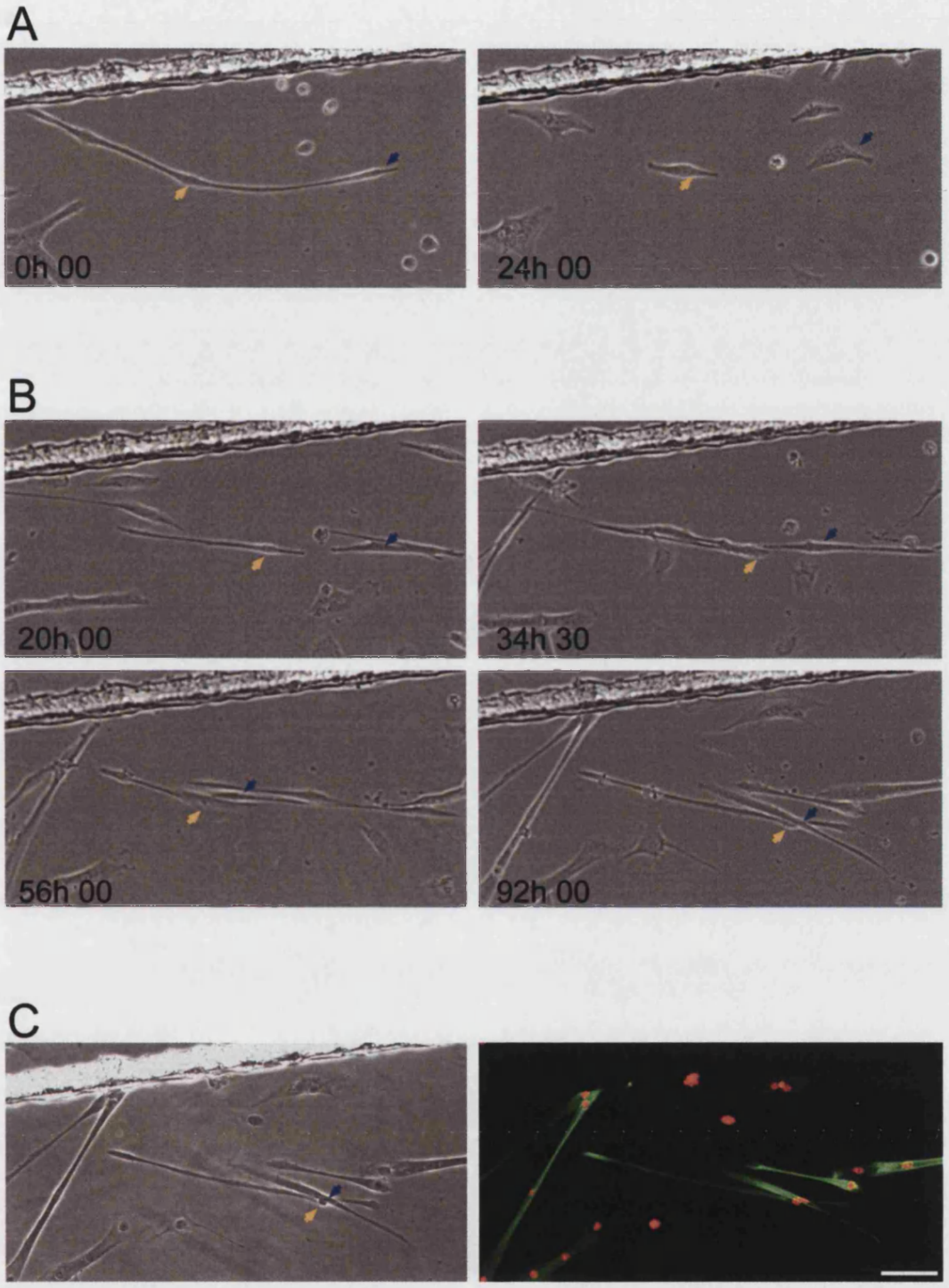


Figure 5-4: MHC expression in mononucleate progeny followed by time-lapse. Purified Pmi28 myotubes were treated with 15μM 109 for 24 hours, then washed with D-PBS and maintained in growth medium for a further 96 hours, followed by fixation and staining for MHC. Chosen fields were imaged by time-lapse microscopy throughout, individual myotubes were imaged at 5minute intervals during 109 treatment, and the resulting mononucleate progeny at 20minute intervals over the subsequent four day period. Selected stills from a time-lapse movie are shown here at the start and immediately following 24 hour treatment with 109 (A), to illustrate the formation of two mononucleate cells from a single myotube; the same field is shown at selected time points over the 96 hour period in growth medium (B), and in phase contrast and fluorescence following fixation and staining for MHC (green) and nuclear staining with propidium iodide (red) (C). The two mononucleate progeny are marked with arrows at each stage, both cells have clearly retained expression of MHC. Scale bar = 100μm.

5.2.2.2 Distribution of centriole markers in myoblasts and myotubes

The centrosome is the microtubule organising centre (MTOC) in mononucleate myoblasts as in many other cell types and in interphase cells is usually located close to the nucleus. The centrosome consists of two centrioles; each duplicates once every cell cycle giving rise to the two centrosomes that organise the poles of the mitotic spindle, enabling the cell to segregate its chromosomes and complete mitosis. During myogenic differentiation, myoblasts withdraw from the cell cycle and therefore no longer have a need for many components of the cell division machinery. Reports suggest that some centrioles are lost and those which remain no longer act as the primary MTOCs of the cell. The coordinated regulation of centrosomes and the cell cycle suggested that the presence or absence of centrioles in mononucleates produced by cellularisation may have consequences for the fate and behaviour of these cells and provided an additional aspect to their characterisation.

In order to study the distribution of centrioles in Pmi28 cells, antibodies to two centriolar antigens, γ-tubulin and pericentrin, were used. γ-tubulin is a well-established component of the centrosome and antibodies to γ-tubulin are often used to identify centrioles by immunohistochemistry. Pericentrin is part of the pericentriolar material (PCM) and is closely associated with γ-tubulin (Dictenberg et al., 1998). Pmi28 mononucleates and purified myotubes were fixed and double-stained with both antibodies (Figure 5-5). In the majority of mononucleates, γ-tubulin could be seen as a pair of brightly staining spots in the cytoplasm, often close to the nucleus. Figure 5-6 demonstrates that in a population of proliferating myoblasts the majority of cells contained two γ-tubulin foci. Pericentrin colocalised with the γ-tubulin spots but was visible as a more diffuse cloud of pericentriolar satellites in the PCM surrounding the centrioles. Although not all cell types show the localisation of centriolar proteins in such satellites, mouse myoblasts have previously been reported to possess cytoplasmic granules surrounding the centrioles and containing, among other proteins, pericentrin (Dammermann and Merdes, 2002). In the myotubes, γ-tubulin foci were still visible but stained much more weakly; again, pericentrin colocalised with γ-tubulin, but also with greatly reduced intensity. In addition, circumnuclear distribution of pericentrin was seen for all nuclei in myotubes, γ-tubulin staining was restricted exclusively to the spots within the cytoplasm.

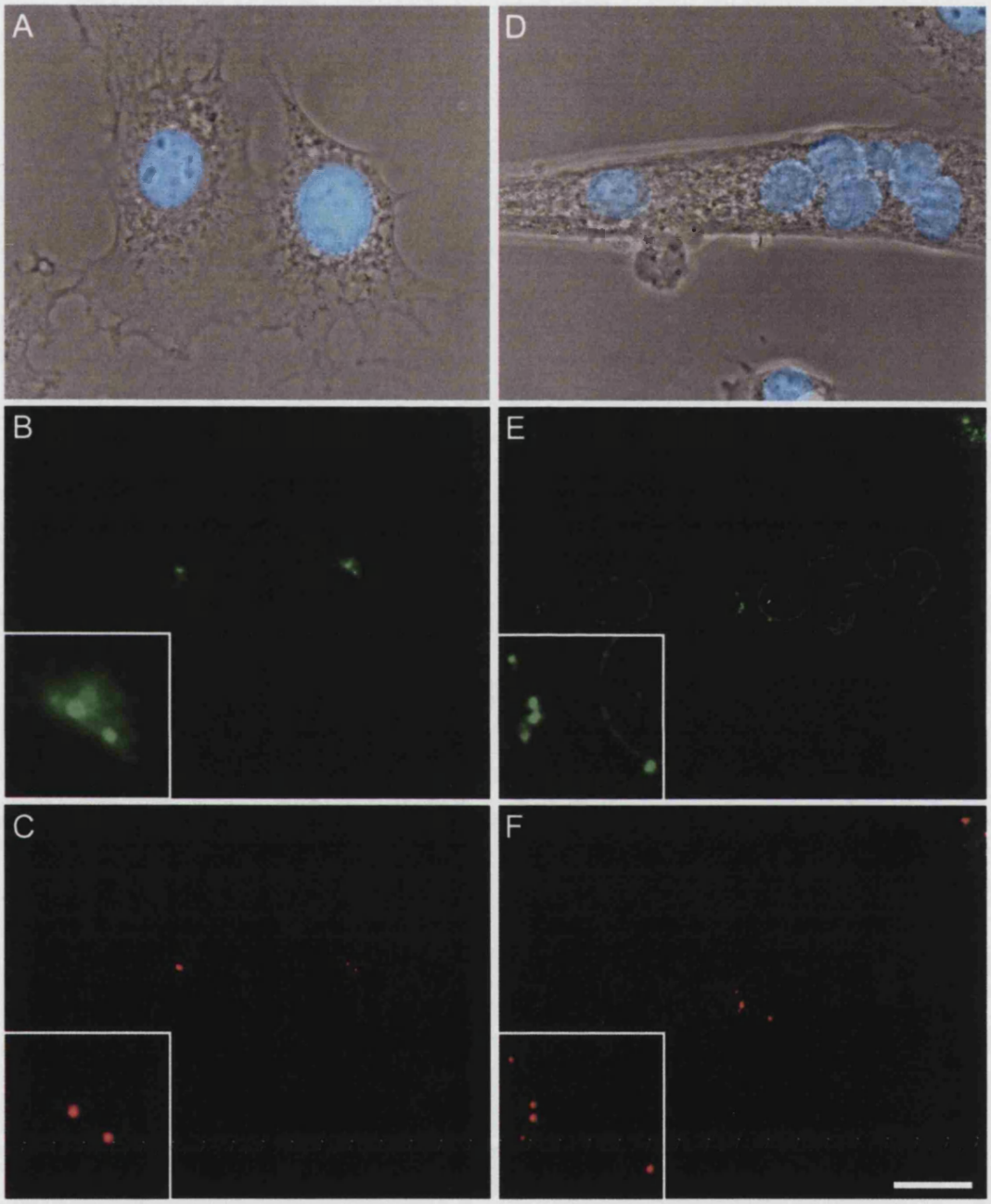


Figure 5-5: Immunohistochemical staining of Pmi28 mononucleates and myotubes for centriolar antigens. Mononucleates (A-C) and myotubes (D-F) were fixed and stained for pericentrin (B, E) and gamma-tubulin (C, F), nuclei were stained with hoechst (blue in A and D). The fields shown in phase contrast images A and B, correspond to the immunofluorescence images directly below. In-lays show enlarged regions of the same image. Centrioles are detected as points of colocalisation of pericentrin and gamma-tubulin; in mononucleate cells, gamma-tubulin is seen as two closely associated spots while pericentrin also stains pericentriolar satellites surrounding the centrioles. In myotubes, centrioles are still positive for both markers although stain less intensely and can be observed as single spots or in clusters, in addition, pericentrin is redistributed to surround all nuclei within the myotube. Scale bar = 20µm.

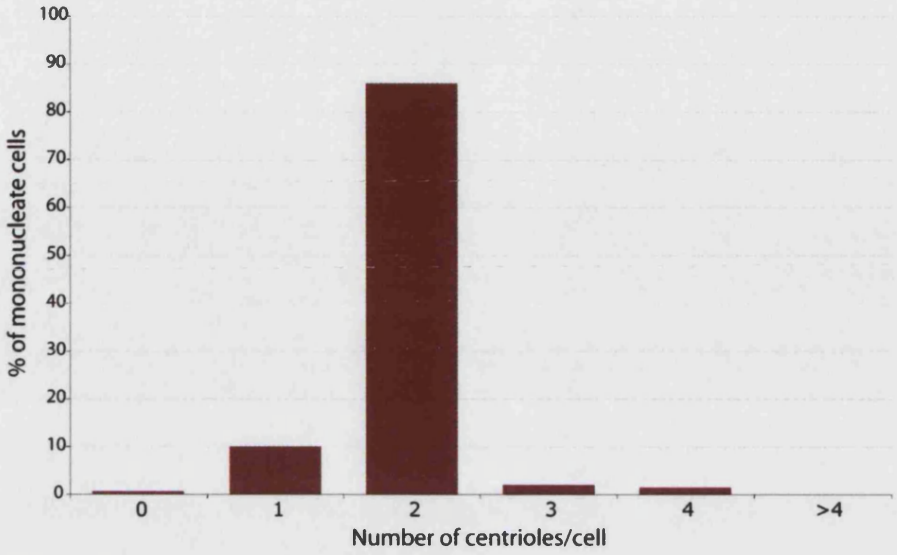


Figure 5-6: Quantitation of centrioles in Pmi28 mononucleates. Mononucleate cells were stained for gamma-tubulin and the number of foci in cells from randomly chosen fields was counted. A total of 400 cells were counted and only cells with a single nucleus were included. The majority of cells contained two gamma-tubulin foci, usually seen as a pair of dots close to the nucleus.

To determine whether circumnuclear localisation of pericentrin was associated with differentiation, Pmi28 mononucleates were cultured in differentiation medium for 24 hours then fixed and stained for both MHC and pericentrin. Figure 5-7 shows that in MHC positive cells pericentrin appears to be localised to the nucleus while in MHC negative cells it remains associated with the centrosome. Figure 5-7 D shows quantitation of the association between MHC expression and pericentrin localisation, and suggests that there is a correlation between differentiation and the redistribution of the PCM component pericentrin, to the nuclear surface.

Other changes to centriolar organisation observed in myotubes were that centrioles no longer remained associated in pairs, as they were most commonly seen in mononucleates, and were not necessarily associated with nuclei. Instead, centrioles were seen as isolated foci or sometimes as clusters of centrioles, as in Figure 5-5, and appeared to be randomly distributed in the cytoplasm. In addition, the ratio of two centrioles per nucleus looked to have been reduced suggesting that centrioles were being lost.

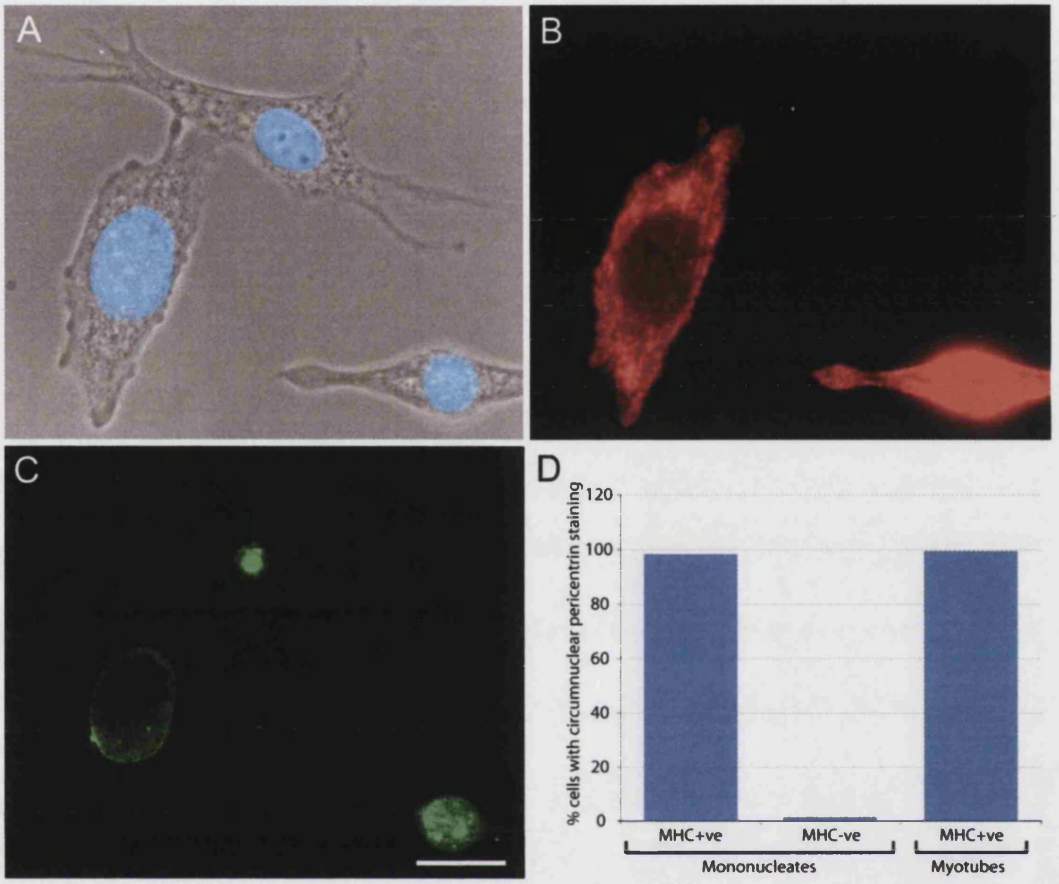


Figure 5-7: Correlation of circumnuclear localisation of pericentrin and myogenic differentiation in Pmi28 cultures. Phase contrast (A) and immunofluorescence of mononucleate cells double stained for MHC (B) and pericentrin (C), nuclei were stained with Hoechst (blue in A). Pericentrin is localised to the nuclear surface only in MHC positive cells. Quantitation of this observation (D), shows that the percentage of MHC positive mononucleates and myotubes in which nuclei are stained positive for pericentrin exceeds 98%, while in MHC negative mononucleates less than 2% show circumnuclear pericentrin, indicating that localisation of the antigen is associated with biochemical differentiation. Scale bar = 20μm.

In order to confirm this, the centrioles in myotubes were counted. As γ-tubulin staining of centrioles was much weaker in myotubes than in mononucleates, purified myotubes were fixed and double-stained for γ-tubulin and pericentrin. Centrioles were identified as points of colocalisation for both markers in order to make detection of centrioles easier and less ambiguous. Pmi28 cells were cultured at high density in differentiation medium for three days to induce fusion, then were purified and incubated for a further 24-48hrs in differentiation medium before fixation (termed 'old' myotubes). The number of centrioles and nuclei in myotubes were counted. These counts were compared with those from cells that had been allowed to fuse for only one day (termed 'young' myotubes) before purification and then incubated for just 24 hours prior to fixation. Figure 5-8 shows that for the culture fused for one day, the results are clustered close to, or on the

line denoting a 2:1 ratio while in cultures fused for longer, the results are more scattered. This suggests that centrioles are lost from myotubes over time; in the 'young' culture, fused for only one day, 58.3% of the myotubes contained two centrioles for every nucleus while in cultures allowed to differentiate for longer this number was only 6.3%.

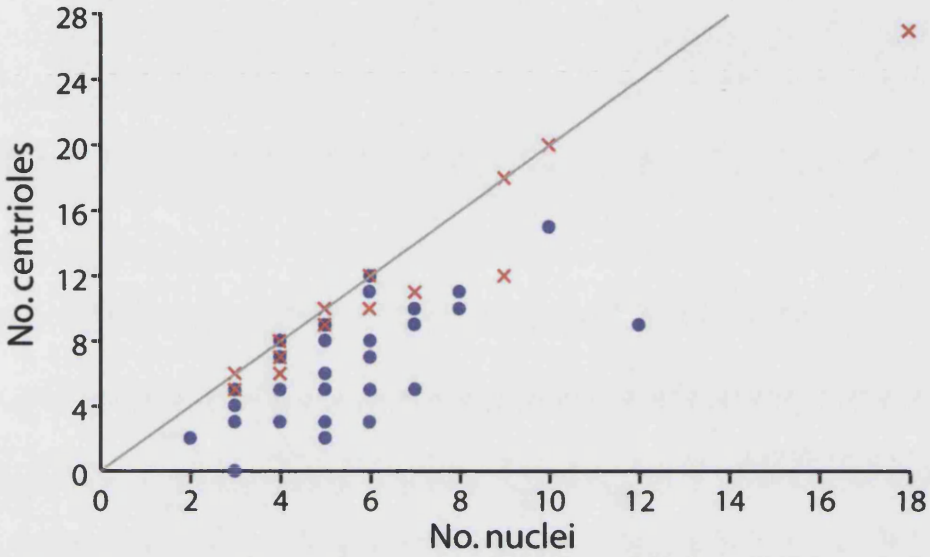


Figure 5-8: Comparison of centriole counts in 'young' and 'old' myotubes. Cultures of 'young' myotubes were obtained from Pmi28 culture induced to differentiate for 24 hours followed by purification and a further 24 hours incubation to allow the myotubes to adhere. 'Old' myotubes were induced to differentiate for three days followed by purification and a subsequent 24-48 hour incubation period. Both populations were fixed and stained with antibodies against pericentrin and gamma-tubulin, nuclei were stained with hoechst. Only foci which stained positive for both markers were counted as centrioles. The results are expressed as the number of centrioles per nucleus for 24 'young' myotubes (×) and 32 'old' myotubes (●), the line represents a 2:1 ratio. In myotubes induced to differentiate for just one day, the cell counts are clustered close to the 2:1 ratio while in myotubes cultured for longer, this relationship between nuclei and centrioles is lost indicating a loss of centrioles over time.

To determine whether treatment with 109 directly affected the number and organisation of centrioles, myotubes were treated as for the time-lapse experiments described above, before fixing the cells and counting the number of centrioles. Myotubes were purified after differentiation for three days, maintained in differentiation medium for a further 24-48 hours, then treated with 15µM 109 for 24 hours, followed by four days in growth medium. Control dishes were prepared in parallel, omitting 109 treatment. Figure 5-9 shows the results for centriole counts in 109-treated myotubes and illustrates a striking difference from the results displayed in Figure 5-8; in 109-treated myotubes the ratio of centrioles to nuclei exceed 2:1. This suggests that the random distribution of centrioles seen in the myotubes is not reorganised during cellularisation, giving rise, in some cases,

to myotube fragments with more than two centrioles per nucleus. Only a single control myotube in Figure 5-9 was observed to possess a greater than 2:1 ratio.

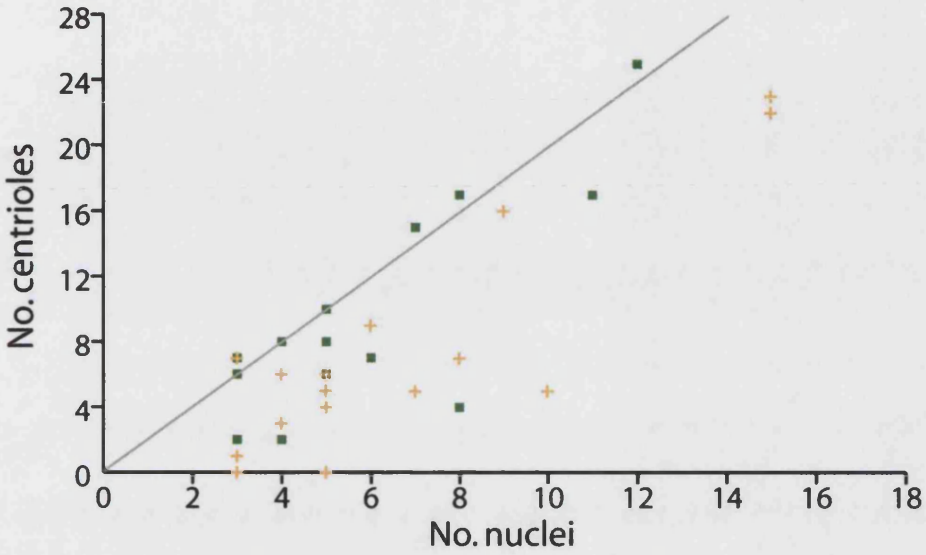


Figure 5-9: Comparison of centriole counts in 109 treated and control myotubes. Pmi28 cultures were induced to differentiate for three days, then purified and treated as for the time-lapse experiments shown in Figure 5-3 and Figure 5-4. Purified myotubes were treated with 15μM 109 (▪), or DM alone (+) for 24 hours, then maintained in growth medium for a further four days. Cells were fixed and stained with antibodies against pericentrin and gamma-tubulin, nuclei were stained with hoechst. The numbers of centrioles and nuclei were counted in 16 control myotubes and 22 myotubes treated with 109, the line represents a 2:1 ratio. In control myotubes, the number of centrioles per nucleus varies but exceeds a 2:1 ratio in only a single instance. Following 109 treatment the ratio is more varied and in several myotubes lies above the line, this suggests that, centrioles are distributed randomly in the cell, and during treatment with 109 are not actively segregated between the resulting myotube fragments.

Observation of myotubes following treatment with 109, and staining for γ-tubulin and pericentrin, revealed no apparent changes in the organisation or location of centrioles within the cells. The average ratio and standard deviation for counts made under the different conditions are compared in Table 5-3. The 'young' one day myotube cultures display the mean count closest to 2, and have the lowest standard deviation. The myotubes maintained in culture for the longest have the lowest mean and also show higher standard deviation.

Table 5-3: Summary of average centriole:nucleus ratios in 109 treated and control myotubes.

	See **Figure 3-9**		See **Figure 3-10**	
	'Young' myotubes	'Old' myotubes	Control	109 treated
mean centriole/nuclei ratio	**1.85**	**1.22**	**1.02**	**1.06**
standard deviation	0.20	0.48	0.65	0.48
n	*24*	*32*	*16*	*22*

Purified myotubes were treated as described in the legends for Figures 5-8 and 5-9, the results depicted graphically are summarised here. The decrease in the average ratio with time indicates that centrioles are progressively lost from myotubes, this process appears to be unaffected by 109 treatment.

These results support the premise that centrioles are lost from myotubes in culture over time, even in those myotube fragments which may have gained an enhanced ratio of centrioles to nuclei following fragmentation with 109.

5.2.2.3 Distribution of centriole markers in mononucleates resulting from cellularisation of myotubes, and followed by time-lapse

As described earlier, time-lapse microscopy was used to capture multiple cellularisation events and then follow the resulting mononucleates for four days. At the end of four independent experiments, the cells were fixed and stained for both γ-tubulin and pericentrin. These four experiments gave rise to a total of 43 mononucleates, which were stained and examined for the presence of centrioles. In all cases, the reliability of staining was verified by checking that accompanying myoblasts were positive for the markers. Only a single mononucleate resulting from cellularisation of Pmi28 myotubes by the action of 109 contained centrioles. The cell contained three small, barely detectable spots to which both γ-tubulin and pericentrin were localised. That the cell contained three centrioles instead of the two normally present in mononucleate myoblasts, supports earlier observations that the distribution of centrioles between myotube fragments appears to be a random process and does not depend on the number of nuclei.

5.2.3 Induction of adipogenic differentiation in Pmi28 and C2C12 myoblasts

Pmi28 myoblasts were cultured under adipogenic conditions to assess the multipotency of the cell line, with a view to possibly determining the potentiality of the mononucleate progeny produced during cellularisation, and whether they possess an enhanced ability to undergo differentiation along alternative lineages. Pmi28 myoblasts, C2C12 myoblasts and LiSa-2 cells were plated onto collagen-coated 35mm dishes at approximately 60-80% confluence. The LiSa-2 cell line is derived from a poorly differentiated human liposarcoma and readily undergoes differentiation, accompanied by accumulation of cytoplasmic lipid droplets, to form fat cells, in the presence of adipogenic medium. C2C12 is a murine myogenic cell line. All three cell lines were cultured in a commercially available adipogenic medium (Cambrex) containing insulin, dexamethasome, indomethacin and isobutylmethylxanthine for a total of 15 days. Cells

were then fixed and stained with nile red to detect the presence of intracellular lipid droplets (Greenspan et al., 1985).

During the 15 days in culture, the cells were photographed at regular intervals to monitor their differentiation. Figure 5-10 shows the three cell lines at 0, 5, and 15 days.

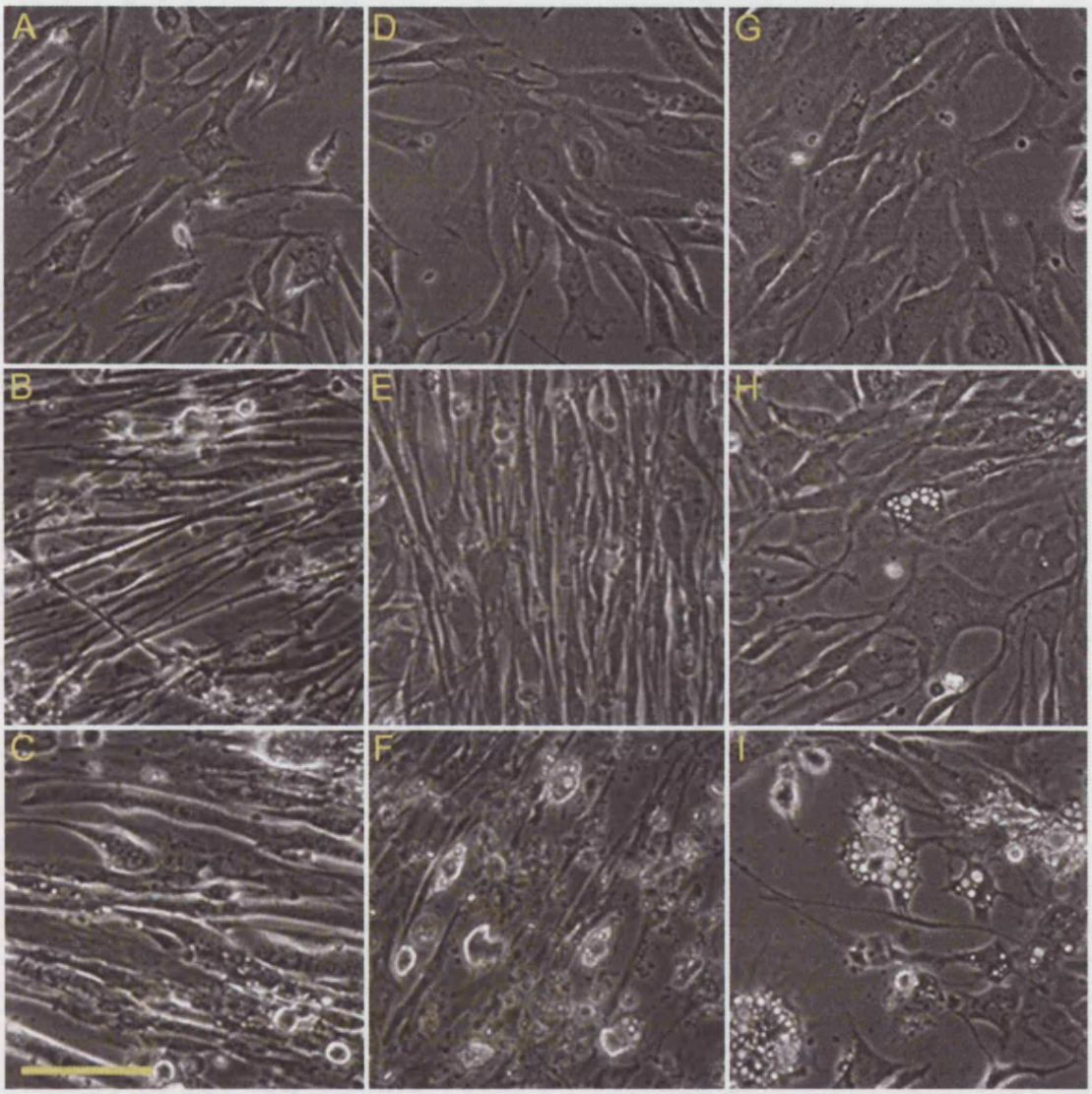


Figure 5-10: Culture of Pmi28, C2C12 and LiSa-2 cells in adipogenic medium. Mononucleate cells were grown to ~60% confluence then cultured for 15 days in adipogenic medium, live cells were photographed using phase contrast optics, at the start (A, D, G), after 5 days (B, E, H) and after 15 days (C, F, I). Pmi28 mononucleates (A-C) elongated and began to fuse to form multinucleate myotubes, although cell death was much higher than during culture in myogenic medium. Cells of the mouse myogenic cell line, C2C12 (D-F), initially continued to proliferate reaching 100% confluence by 5 days. These cells failed to undergo myogenic differentiation but underwent widespread cell death, of the cells remaining after 15 days, a small proportion appeared to have accumulated lipid droplets. The human LiSa-2 cell line (G-I), showed some evidence of lipid accumulation after just 5 days and by the end of the culture period, around 50% of cells contained significant numbers of lipid droplets. Scale bar = 100µm.

In both the C2C12 and Pmi28 cell lines there is extensive cell death; unhealthy cells and cell debris can be seen. However, after 10 days in culture some lipid droplets are detectable in the remaining C2C12 mononucleates and by day 15 a number of cells containing large, round lipid droplets are visible (see also Figure 5-11 D). In the LiSa-2 cell line, cells have also accumulated lipid by the end of 15 days but in these cells the accumulation takes the form of clusters of many small droplets rather than the fewer large droplets in the C2C12 cells (Figure 5-11).

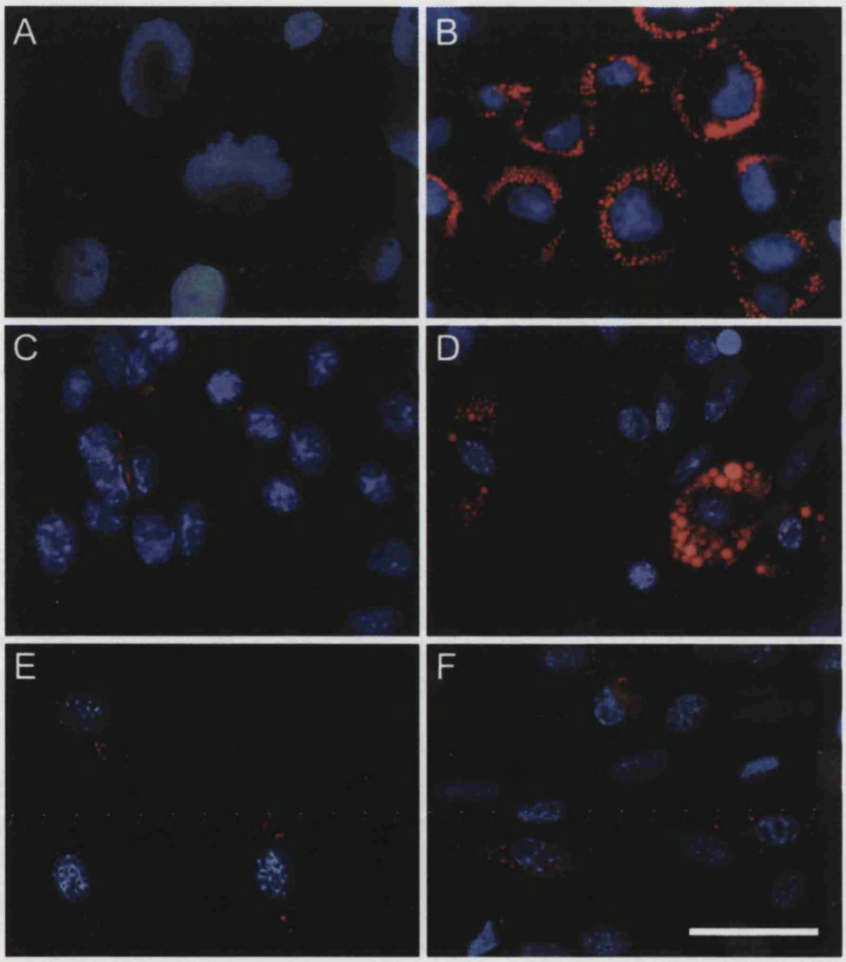


Figure 5-11: Nile red staining of cell lines after culture in adipogenic medium. Mononucleate cells of the Pmi28, C2C12 and LiSa-2 cell lines were cultured for 15 days in adipocyte differentiation medium to assess the cells ability to undergo adipogenic differentiation. At the end of the 15 day culture period cells were stained with nile red to detect intracellular lipids (red) and Hoechst to show the nuclei (blue). Control populations of LiSa-2 (A), C2C12 (C) and Pmi28 (E) mononucleates in standard growth medium were stained in parallel and were all negative for lipid droplets. After 15 days in adipocyte differentiation medium, LiSa-2 (B) and C2C12 (D) cells contained detectable accumulation of lipid droplets indicative of adipogenic differentiation, while Pmi28 cells (F) remained negative when stained with nile red. Scale bar = 50μm.

Many of the LiSa-2 cells have enlarged or deformed nuclei but there is little cell death. In contrast, there is no evidence of adipogenic differentiation in the form of lipid droplets in the Pmi28 cells (Figure 5-11); instead these cells appear to have continued to undergo limited myogenesis. The cells have begun to elongate and align after 5 days and a number of myotubes are visible by day 15 (Figure 5-10).

5.3 DISCUSSION

Earlier experiments have established the production of mononucleate cells from multinucleate myotubes by the action of 109 but so far have not investigated the consequences this cellularisation event may have for the fate of these mononucleates. Reports in the literature have focussed on the proliferative capacity and potentiality of mononucleates arising from the fragmentation of multinucleate muscle cells by a variety of mechanisms (see Chapter 1). Conditional expression of Msx-1 in murine C2C12 myotubes was reported to lead to cellularisation of a proportion of these myotubes, giving rise to mononucleate cells that were capable of division (Odelberg et al., 2000). It has also been claimed that cells formed by cellularisation of C2C12 myotubes with myoseverin, were capable of division (Perez et al., 2002; Rosania et al., 2000). During newt limb regeneration, the cells contributed to the blastema by dedifferentiation of implanted myotubes are mononucleate and capable of division (Kumar et al., 2000; Lo et al., 1993), while recent work in the urodele system *in vitro* has provided insight into an endogenous programme of cellularisation in primary axolotl myofibres; these myofibres undergo spontaneous budding when dissociated and maintained in culture, giving rise to mononucleate cells, which appear to be capable of proliferation (Kumar et al., 2004).

Although my earlier microarray studies provided no evidence of dedifferentiation in myotube cultures during cellularisation by 109, either by down-regulation of muscle-specific transcripts or activation of genes required for re-entry into the cell cycle, cellularisation by 109 nonetheless represents a reversal of the mononucleate to multinucleate transition which is a characteristic of muscle differentiation. In light of the reports cited above it was therefore of great interest to determine whether mononucleates generated by cellularisation of Pmi28 myotubes through the action of 109, were also

capable of division, and potentially to assess whether cellularisation might be sufficient to trigger a wider programme of dedifferentiation, including cell cycle re-entry.

Initial attempts to determine whether mononucleate progeny were undergoing division were carried out by plating purified myotubes into grid squares and photographing those squares in which a cellularisation event occurred, in response to 109, at 24 hour intervals. The main problem was in definitively identifying the mononucleate of interest at successive timepoints; during a 24 hour period cells changed both shape and location. The scored grid lines proved an insufficient barrier to movement of cells between the squares, which meant that contaminating myoblasts were able to invade fields under study. Cells expressing a nuclear localised eGFP were used to try and clarify events, but although no significant difference was seen in fragmentation frequency or mononucleate fate in the presence of a nuclear label, their use was abandoned for fear that exposure to UV light may damage DNA and affect the cells ability to divide.

The problems inherent in the design of these experiments limited the conclusions that could be drawn from this data alone. It was only really possible to obtain information from grid squares where there was little cellular movement which by definition identifies squares where cells did not divide and so biased the experiment towards collection of data for negative aspects of cell behaviour. However, these experiments were an early indication that a large proportion of cells did not undergo division, a suggestion that was strengthened by subsequent work using more rigorous techniques. In addition, this approach provided information from 415 myotubes about the frequency of fragmentation events, revealing that around 50% of myotubes, with a typical elongated morphology, underwent fragmentation to form smaller myotubes and/or mononucleates.

The absence of incorporation of BrdU into MHC positive mononucleates supported the initial suggestion that mononucleates produced following cellularisation of myotubes with 109 were not able to divide. Although entry into S phase is not a guarantee that cells will go on to enter mitosis, incorporation of BrdU into these mononucleates would represent, if not quite evidence of cell division, then at least evidence of cell cycle re-entry and reversal of one important aspect of terminal differentiation in muscle cells. During myogenic differentiation, mononucleate myoblasts withdraw from the cell cycle

when in G1 by a process involving the retinoblastoma protein (as described in Chapter 1). This cell cycle arrest is irreversible in mammalian cells without genetic manipulations, such as inactivation of the Rb gene or infection with viral oncoproteins, and is a characteristic of terminal differentiation in mature muscle. The nuclei in cultured newt myotubes however, have been shown to re-enter S phase in response to serum while the nuclei in mammalian myotubes remain refractive to all growth factors and serum components tested (Tanaka et al., 1997). While the significance of this result in the context of regeneration is not yet fully understood, it has been suggested that it may provide valuable clues to understanding the disparity in regenerative capacity between the urodeles and mammals.

When the effect of 109 on S phase re-entry in myotubes was examined, experiments consistently showed that BrdU incorporation both during and after 109 remained comparable with that in control myotubes; 109 does not induce S phase re-entry in mammalian myotubes. The slightly higher levels of BrdU incorporation in untreated myotubes is probably due to the continued division and fusion of cells in the control culture. In the absence of 109, mononucleate cells continue to divide, thus incorporating BrdU, and fuse as the culture becomes more confluent. Because of this, the number of cells incorporating BrdU, in both 109-treated and control cultures, is higher in experiments where the cells are stimulated to divide in growth medium.

It is possible that the 24 hour period following 109 treatment used in these experiments may not be sufficient to completely remove inhibitory mechanisms and reinitiate the cell cycle in these newly formed cells. However, previous reports documenting the stimulation of cell cycle re-entry in mammalian myotubes, in Rb null cells or following retroviral infection (see Chapter 1), or in mononucleates arising from cellularisation events (Odelberg et al., 2000; Perez et al., 2002) observed positive results within 24 hours, suggesting this is an appropriate time scale.

While the experiments quantitating incorporation of BrdU into MHC positive mononucleates provide indirect evidence to suggest that the cells generated by cellularisation of myotubes do not re-enter the cell cycle, they reveal little information about the behaviour of the mononucleates and ultimately I turned to time-lapse

microscopy to provide a more comprehensive analysis. One crucial advantage in using the time-lapse system to detect cell division is that it does not rely on the retention of MHC by the mononucleates under study. This removes the criticism that the cells more likely to undergo division will have lost MHC and thus escape detection, resulting in a biasing of the assay towards cells that will not divide because they retain markers of differentiation. The time-lapse experiments deal exclusively with mononucleates generated by cellularisation and so are indifferent to the presence of background mononucleates in the culture.

Time-lapse is a very powerful tool for following cell morphology and behaviour over time. Given that only a single dish of cells can be analysed at a time, and the length of time for which I wished to run my experiments, i.e. five days, the application of a motorised stage was a valuable part of the experimental design. It allowed the examination of significant numbers of mononucleates, adding statistical weight to the study and enabling conclusions to be made with greater confidence.

Of 34 mononucleates followed by time-lapse microscopy for 48 hours in growth medium following 109-induced cellularisation, and a further 112 mononucleates followed for 96 hours, no cases of cell division were observed. Approximately 70% of these cells survived as mononucleates and rapidly obtained an elongated shape reminiscent of post-mitotic myoblasts. When stained for the presence of MHC, all mononucleate progeny analysed retained the marker. The continued expression of MHC after four days in growth medium suggests that these cells have not undergone dedifferentiation following cellularisation and the appearance of striations in the MHC staining in some of these cells implies that the cells are continuing to undergo further differentiation even after cellularisation. In agreement with the results of the earlier microarray analysis, the fragmentation of myotubes into mononucleates by the action of 109 does not appear to have affected progression of the myogenic programme.

Myoseverin was reported to stimulate DNA synthesis in differentiated myotube cultures, in a serum-dependent manner, following a period of treatment with the compound (Perez et al., 2002; Rosania et al., 2000). The authors suggested that such data was indicative of proliferation of the mononucleate cells derived from myotubes by the action of

myoseverin. However, it should be noted that no analysis at a single cell level was reported and BrdU incorporation in the entire culture was measured using an ELISA type absorbance-based assay system which does not identify the location of positive nuclei and provided no indication of whether entry into S phase was occurring in resident myoblasts, multinucleate myotubes, or mononucleates generated by cellularisation. My assessment of BrdU incorporation both during and after cellularisation looked specifically at S phase re-entry in the different populations of cells present in the culture making clear distinctions between mononucleate and multinucleate cells, and between MHC positive and negative cells, and the use of time-lapse microscopy to follow the progeny resulting from cellularisation with both 109 and myoseverin directly challenges the claims made in this earlier work that the resulting mononucleates are capable of division. Inclusion in my analysis of two independent time-lapse experiments conducted with myoseverin dismisses the possibility that the structural differences between 109 and myoseverin are relevant to their mechanism of action.

Nocodazole has been used to synchronise cultures of proliferating cells (Zieve et al., 1980) and I propose that this may provide an alternative explanation for the increased entry into S phase reported following myoseverin treatment. While mononucleates in both treated and untreated cultures will respond to the introduction of high serum, there may be a lag period, and if cells begin to respond towards the end of the 24 hour period of BrdU incorporation, the response of a pre-synchronised culture entering S phase simultaneously will provide a greater read-out than a culture in which some cells have begun DNA synthesis while others have not yet entered S phase. By consistently referring to a complex culture as a single population, the conclusions to be drawn are limited by the availability of data, and should always be made with an awareness of the inherent restrictions of such an approach.

The combined use of several approaches, observing isolated cells in grid squares, detection of S phase re-entry by BrdU incorporation, and finally the use of time-lapse microscopy to unequivocally follow individual mononucleate progeny, demonstrated that mononucleate cell arising from cellularisation of Pmi28 myotubes by 109, remain viable but do not proliferate; I have discussed above possible reasons for the apparent inconsistency between my work and that reported for myoseverin. A logical next step

was then to ask why these mononucleate cells are unable to divide when the progeny of myotubes undergoing cellularisation by ectopic expression of Msx-1 (Odelberg et al., 2000), or during urodele regeneration in the amputated limb (Kumar et al., 2000; Kumar et al., 2004; Lo et al., 1993), appear to be capable of extensive proliferation. What aspects of myogenic differentiation are reversible in these cases but cannot be reversed by the action of 109?

As mentioned previously, the centrosome, a structure at the heart of microtubule organisation in almost all mammalian somatic cell types and with roles in the nucleation of interphase arrays and formation of the mitotic spindle, appears to be lost during myogenesis. These organelles, replicated faithfully every cell cycle along with the cells genome, and so clearly implicated in various aspects of cell division, are no longer required by a post-mitotic cell and appear to be degraded in multinucleate myotubes following fusion, being undetectable in mature myofibres (see Chapter 1). Centrioles arise by duplication of existing centrioles and so the loss of such a structure lends an additional, and seemingly irreversible aspect to the process of differentiation. That an absence of centrosomes may at least contribute to the cells inability to undergo cell division is an attractive hypothesis and was subsequently investigated by the detection of centrosomes in Pmi28 myotubes and the mononucleate progeny following 109-induced cellularisation.

The presence of centrioles in proliferating mononucleates, and their progressive loss from myotubes over time, was confirmed in the Pmi28 cell line by the staining of cells with γ-tubulin and pericentrin, both integral components of the mammalian centrosome (Bornens, 2002; Dictenberg et al., 1998). Double staining with two centriolar markers made detection of centrosomes easier and more reliable, given the microscopic nature of such organelles.

In the majority of Pmi28 myoblasts, γ-tubulin staining was visible as two distinct but closely associated foci probably representing the two parental centrioles during G1 and later two centrosomes each comprised of a duplicated centriole pair. A study of centrosome movement in a number of mammalian cell lines demonstrated that the two parental centrioles are independent units during most of interphase but are maintained in

close proximity by the normal dynamics of the microtubule cytoskeleton and the tensions created by microtubule arrays nucleated by both centrioles (Jean et al., 1999). The process of centriole duplication begins at the onset of S phase with the budding of pro-centrioles, elongation of the daughter centrioles continues throughout S phase and into G2. However, splitting or disorientation of the centriole pair can occur much sooner, as early as telophase (Piel et al., 2001), which may explain the presence of four centrioles in some myoblasts.

Cell cycle withdrawal during myogenic differentiation occurs when the cells are in G1, at a time when the cell contains a single centrosome consisting of a pair of centrioles. Following fusion myotubes should therefore contain two centrioles for every nucleus. This correlation is present in so-called 'young' myotubes, purified after just one day of fusion, a finding that also validates the double staining approach used here to identify points of colocalisation of pericentrin and γ-tubulin as individual centrioles. The separation of the centriole pairs within the myotube, and the random distribution in the cytoplasm described here, is supported by early work on the fate of MTOCs in myotubes (Tassin et al., 1985). A loss of centrioles from myotubes over time is clearly seen in my experiments with Pmi28 cells, by comparing myotubes after only one day in differentiation medium, with those allowed to differentiate for longer, and has also been well documented in a number of earlier studies, in primary muscle cultures, employing both immunofluorescence (Connolly et al., 1986; Tassin et al., 1985) and electron microscopy (Przybylski, 1971) techniques.

An additional observation was the striking difference in localisation of pericentrin between mononucleate myoblasts and multinucleate myotubes. The circumnuclear distribution of pericentrin following differentiation has been reported previously (Musa et al., 2003; Tassin et al., 1985), and appears to correlate with a redistribution of MTOC activity away from the centrosome, to the region surrounding the nucleus. Microtubule re-growth, following brief treatment with nocodazole to depolymerise all existing microtubules, occurred from a single distinct point in mononucleate cells, radiating outwards, while in myotubes microtubule re-growth occurred primarily from around the nucleus.

The significance and mechanism of this redistribution of pericentrin and MTOC activity is not known. In most cell types, the centrosome is positioned close to the nucleus and in eukaryotic cells which do not possess centrioles, MTOC activity is often localised around the nucleus. In plant cells, which lack centrosomes, the nuclear surface has been shown to be a site of microtubule nucleation (Canaday et al., 2000). The close association between centrioles and nuclei suggests that the PCM may have an affinity for the nuclear envelope and as centrioles are lost following myoblast fusion the PCM becomes redistributed around the nucleus. Interestingly, this pattern of pericentrin staining is also seen in mammalian osteoclasts. Mature osteoclasts are post-mitotic, multinucleate cells and also display a dramatic decrease in the number of centrioles following fusion; in mammalian osteoclasts, 86% of cells were reported to have lost all centrosomal MTOCs (Mulari et al., 2003). Occasional centrosomes, labelled with γ-tubulin, were observed singly or in clusters but only very rarely did they demonstrate nucleation of microtubules; instead, microtubules were nucleated from the vicinity of the nuclei. The results described for mammalian osteoclasts closely parallel the findings described in this chapter for mammalian myotubes. In returning to the proposition that centrosomes are required for cell division, it is noteworthy that avian osteoclasts, which do not appear to lose centrioles, are also capable of undergoing a reversal of the mononucleate to multinucleate transition, generating mononucleate cells by a mitosis-independent budding mechanism (Solari et al., 1995).

The centriole counts obtained from myotubes fixed immediately after treatment with 109 provided initial evidence that there is no mechanism to segregate centrioles along with nuclei during cellularisation and that the number of centrioles, and indeed the number of nuclei, received by a given myotube fragment during treatment with 109 is stochastically determined. Under these circumstances, the number of centrioles per nucleus in a myotube fragment may exceed the 2:1 ratio. In agreement with a role for centrosomes in division, of 43 mononucleate progeny, arising by cellularisation of Pmi28 myotubes with 109 and followed by time-lapse, only one cell contained detectable γ-tubulin and pericentrin positive foci, and all 43 failed to divide. This single cell received three centrioles. Together, these results provide compelling evidence that the segregation of centrioles, and potentially other organelles required by the cell, during cellularisation by 109 is a random process. That only a single mononucleate contained any detectable

centrioles is perhaps surprising given that at this stage, some centrioles are still present in the myotubes (Figure 5-9). One reason for this may be that the nuclei within myotubes most likely to form mononucleates on treatment with 109, are often located in narrow regions of the cell and so do not contain much cytoplasm when they break away from the body of the myotube. It is possible that more of the mononucleates initially received centrioles in the cellularisation process but some of them have been lost with time as appears to happen within myotubes.

In order to assess the significance of these findings, and determine whether the irreversible loss of centrioles during myogenesis, and therefore the absence of centrosomes in mononucleate progeny resulting from cellularisation, contributes to the inability of the cell to divide, it is necessary to consider carefully the role of the centrosome in cell division (Raff, 2001). If the hypothesis is to be considered a valid one, it is essential to establish the importance of these organelles and that centrosomes truly are indispensable for cell division.

The centrosome consists of a pair of centrioles embedded in a matrix known as the pericentriolar material (PCM). Although the centrioles are perhaps the most distinct feature of the centrosome, visible by EM as two cylinders oriented at an angle to one another and surrounded by a cloud of electron dense material, it was established by studying functional centrosomes *in vitro*, that it is in fact this surrounding material, the PCM, that is responsible for the nucleation of microtubules (Gould and Borisy, 1977). More specifically, ring-shaped γ-tubulin complexes were identified in the PCM which appeared to act as the template for nucleation of new microtubules (Hagan et al., 1998). The role of the centrioles in the MTOC is to recruit centrosomal components required to nucleate and anchor the microtubules, and to stabilise the centrosome. That centrioles are crucial to the integrity of the centrosome was demonstrated by the treatment of cells with antibodies to a component of the centrioles, leading to dispersal of the centrosome, although this did not block microtubule nucleation at random sites in the cytoplasm, in agreement with the non-essential role of centrioles in nucleation (Bobinnec et al., 1998). Interestingly, in light of comments made above regarding the association of MTOC activity and the nucleus, the scattered pericentriolar material in this experiment was often observed around the nucleus.

During mitosis the microtubule cytoskeleton is rearranged to form the highly dynamic bipolar spindle required to separate the two sets of chromosomes prior to cytokinesis, with the two centrosomes positioned at the spindle poles. However, two notable exceptions to this are higher plant cells and meiotic cell division in most animal systems, which suggest that centrosomes are not an absolute requirement for spindle formation. Centrioles are completely absent in higher plants although γ-tubulin and many other centrosomal proteins still appear to play a role in microtubule nucleation which occurs from multiple sites on the nuclear surface and in the cortex (Canaday et al., 2000). Meiotic divisions in some animals also occurs in the absence of centrioles; by late oogenesis centrioles have been lost and the first and second meiotic divisions proceed by the formation of functional acentriolar spindles. In most species, the centrioles required for further development are delivered by the sperm upon fertilisation but in the mouse, early cleavage events also take place before the appearance of centrioles, providing a rare example of *de novo* synthesis in the absence of existing centrioles (Szollosi et al., 1972).

In addition to these examples, a mounting body of evidence now challenges the traditional role of centrosomes as essential components of the spindle and suggests that animal somatic cells possess alternative means of forming a functional spindle in the absence of centrosomes. The *Drosophila* cell line 1182-4 was derived from a haploid embryo and lacks centrioles although it is maintained as a dividing cell line (Debec et al., 1982). The work of Heald et al described the use of unfertilised Xenopus egg extracts to demonstrate that addition of artificial chromosomes, in the form of chromatin-coated beads, induced the formation of a focussed bipolar spindle in the absence of centrosomes (Heald et al., 1996). Microtubules were nucleated in close proximity to the chromatin, with random orientation but subsequently went through a process of alignment and bundling, followed by focussing into two poles. Such organisation of microtubules is carried out by a number of microtubule motor proteins, several studies have specifically implicated dynein, a minus-end directed motor, in focussing microtubules at the poles (Baas, 1998; Heald et al., 1996; Karsenti and Vernos, 2001).

So it is possible for motor proteins to compensate for the lack of centrosomes and focus the mitotic spindle; but, if, as these studies suggest, the centrosome really is dispensable for spindle formation then why has it been so well conserved and why is it consistently

located at the spindle pole in cells which contain centrosomes? The answer appears to be that the centrosome has a number of other functions within the cell (Rieder et al., 2001). Acentrosomal spindles characteristically lack astral microtubules, which are not involved in the spindle itself but radiate outwards and contact the cortex of the cell. It has been proposed that the real function of the centrosome during spindle assembly is the nucleation of this second population of microtubules, which align the spindle with the axis of the cell and maintain its position with respect to the cleavage furrow, through interactions with the cell cortex (Heald et al., 1996; Khodjakov and Rieder, 2001; Rieder et al., 2001). Support for this is available from the high levels of cytokinesis defects observed in cells in which the centrosome has been removed.

The removal of centrosomes from cells *in vitro* has been achieved through laser ablation (Khodjakov et al., 2000; Khodjakov and Rieder, 2001) or microsurgery (Hinchcliffe et al., 2001; Maniotis and Schliwa, 1991). In all cases, cell division failed to occur with the frequency and efficiency of cells containing centrosomes. Where the centrosomes were removed after the onset of S phase, a bipolar, acentrosomal spindle was seen to form in the majority of cells (Hinchcliffe et al., 2001; Khodjakov et al., 2000; Khodjakov and Rieder, 2001), consistent with the existent of alternative cellular mechanisms for spindle formation in the absence of centrosomes, probably involving chromatin and microtubule motors. However, in 30-50% of cases the cells failed to successfully undergo cytokinesis and the cleavage furrow regressed (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001), in these cells, the spindle appeared to have become incorrectly positioned with respect to the cell axis. All acentrosomal spindle poles lacked the astral microtubules implicated in orientating and positioning the spindle for accurate cytokinesis.

It is significant that none of these experiments reported the regeneration of centrioles, this is despite the fact that cells had not been genetically manipulated and still contained all genes for the centrosomal components. This supports the idea that centrioles must arise from a pre-existing template. In recent years, apparent *de novo* synthesis of centrioles has been reported in CHO cells in which centrioles were destroyed by laser microsurgery while the cells were constitutively arrested in S phase by hydroxyurea treatment (Khodjakov et al., 2002). The authors claim that only in this artificially imposed S phase is there sufficient time and appropriate conditions to allow formation of

centrioles. An earlier report had also claimed to observe the spontaneous reformation of centrioles in mammalian cells (Zorn et al., 1979) although the methodology in this study has been criticised, questioning the reliability of the findings. The mechanism of *de novo* generation of centrioles remains unknown.

These experiments using proliferative cell lines in which the centrioles have subsequently been removed, also highlighted another potential role of the centrosome in cell cycle progression. Although some of the acentriolar cells were capable of forming a functional mitotic spindle and completing cytokinesis, they arrested in G1 of the following cell cycle and were unable to progress into S phase; note that centrioles were removed after commitment to S phase in the first cell cycle (Hinchcliffe et al., 2001; Khodjakov et al., 2000; Khodjakov and Rieder, 2001). These studies are particularly pertinent to the consideration of myotubes and the mononucleate progeny following cellularisation, as they suggest that cells which formerly contained centrosomes do not regenerate centrosomes, and while alternative methods can compensate for some functions, for example during spindle formation and mitosis, the cells remain dependent on centrosomes for other aspects of cell cycle progression.

Centrosome duplication is tightly linked to the cell cycle and is initiated at the G1/S transition, along with DNA synthesis. In keeping with this observed coordination, centrosome duplication appears to be controlled through the Rb pathway which regulates the cell cycle; E2F and Cdk2 activity have been shown to be required for centrosome duplication as for DNA synthesis (Lacey et al., 1999; Meraldi et al., 1999). The mechanism by which the absence of centrioles prevents entry into S phase is currently unknown. Although one has yet to be discovered, there may exist a centriole checkpoint, which prevents S phase entry in the absence of centrioles to ensure the efficiency and fidelity of subsequent divisions. The acentriolar *Drosophila* cell line may represent a case where the cells have been relieved of dependence on the checkpoint (Debec et al., 1982). Alternatively, the centrosome may function to recruit and concentrate factors required for cell cycle progression, bringing proteins into contact and facilitating catalysis of essential reactions (Hinchcliffe et al., 2001; Rieder et al., 2001). The observation that cyclin B-cdk1 is found at centrosomes has added some weight to this theory (Hinchcliffe et al., 2001).

The role of the centrosome has expanded and evolved since it was first described by Boveri in 1888. While work has shown that the assembly of a bipolar mitotic spindle, once thought to be its primary function in cells, can occur in the absence of centrioles, the emergence of alternative roles for this organelle ensure its persistence, and despite exceptions the majority of vertebrate somatic cells still seem to require the presence of centrosomes for proliferation.

Returning to issues surrounding myotube cellularisation, and the possible causal relationship between absence of centrioles and lack of cell division, it would seem that in order for mononucleate progeny, derived from multinucleate myotubes through cellularisation, to progress through the cell cycle and proliferate, one of two processes must take place. One option is for the cells to stimulate *de novo* synthesis of centrioles. Although the dogma is that centrioles may only arise by duplication of existing, some rare examples of *de novo* synthesis have been mentioned above in mouse oocytes or during prolonged S phase arrest.

The second option is that cells are able to activate an alternative mechanism for microtubule nucleation and organisation, acquiring relief from the dependence on centrosomes for cell cycle progression, as seen in the 1182-4 *Drosophila* cell line (Debec et al., 1982). Whatever the system employed in mononucleates generated from myotubes by ectopic Msx-1 expression in mammalian cells, or by urodele muscle fibres during limb regeneration, to enable proliferation, it is clearly not activated in response to 109 and does not occur as a direct consequence of cytoskeletal reorganisation and cellularisation. Evidence from cells in which centrioles were physically removed by laser ablation or microsurgery, suggests that cells which formally contained centrosomes, as do myoblasts prior to fusion, are not fully able to employ acentrosomal mechanisms to replace all the functions of the lost organelles. However, these cells were an actively dividing population and did not display the post-mitotic, multinucleate phenotype of mature myotubes.

It appears that myotubes may already possess specific acentrosomal MTOCs, responsible for the nucleation and organisation of the specialised linear microtubule array in myotubes, which, as shown in Chapter 4 through the use of microtubule depolymerising

agents, is required for maintenance of cell shape and integrity. Currently too little is known about the molecular basis of microtubule organisation in acentrosomal myotubes to determine whether such mechanisms could play a role during dedifferentiation and regeneration. It also remains possible that despite lack of detection of centrosomes in myotubes by immunofluorescence (Connolly et al., 1986; Tassin et al., 1985) or EM (Przybylski, 1971; Warren, 1974), the template for centriole generation may remain intact. Centrosome dispersion using antibodies to poly-glutamylated tubulin, a component of centrioles, was reversed once the antibodies were broken down and functional centrosomes reformed (Bobinnec et al., 1998), in contrast to the irreversible loss of centrosomes following their physical removal from the cell. However, there is no evidence at present to suggest that such dispersal of centrioles occurs under physiological conditions.

The result obtained here with Pmi28 cells, where one mononucleate contained residual centrioles following cellularisation, suggests that the centrioles remaining within myotubes are no longer functional. This is supported by their loss over time and the fact that the cell contained three centrioles, suggesting that the organelles are segregated randomly, and mechanisms which normally ensure that each of the two mononucleates produced by cytokinesis receive one centrosome (consisting of one centriole pair) are not applicable here. This work does not entirely dismiss the possibility that if a mononucleate did receive two centrioles in the stochastic distribution of cytoplasmic components during cellularisation, it may then go on and divide. However, there is no evidence from my work that the action of 109 has any effect on the function or localisation of the centrosome or that cellularisation can reverse this aspect of differentiation. If obtaining two centrioles is sufficient for reactivation of centrosome-dependent processes within the mononucleate it is either still inadequate for cell division or is such a rare event that it did not occur in any of the 146 cellularisation events catalogued here through time-lapse microscopy.

Crucially missing from this discussion is information regarding the presence of centrioles in mononucleates derived from cellularisation of mammalian myotubes by induction of Msx-1, or from myofibres in the amputated limb during urodele regeneration. Work from both these systems suggests that the homeodomain protein Msx-1 may be a key

regulator of cellularisation and dedifferentiation. Although a direct role for Msx-1 in regulation of centrosomes has not been reported, expression is associated with proliferating cells and a downstream effect of Msx-1 in the context of post-mitotic, multinucleate myotubes may be the synthesis and recruitment of centrosomal components.

Considering the importance of the centrosome in cell cycle progression and mitosis as discussed above, the hypothesis that the lack of centrioles in mononucleate cells arising by 109-induced cellularisation of mammalian myotubes, may contribute to the cells inability to divide remains a valid one, although other factors are also likely to play a role. Whether the loss of centrioles is reversible or whether a non-centrosomal mechanism can be activated, and the role of Msx-1, if any, in regulating these events remains to be determined.

Finally, in considering the fate of mononucleate progeny I had hoped to determine their potentiality with regard to their continuing commitment to the myogenic lineage or whether, like the cells produced during Msx-1 induced cellularisation, or during urodele regeneration, these mononucleates may posses the capacity for transdifferentiation. The ability to respond to different environmental cues and so take on different lineage fates is a property associated with multipotent precursor or stem cells and can be used as a measure of the degree of commitment of a given cell type. In the context of my studies with 109, I considered the use of adipogenic agents to judge the plasticity of Pmi28 myoblasts and potentially of the mononucleate cells derived from the cellularisation of Pmi28 myotubes. Pmi28 cells in a proliferative state were maintained in an adipogenic medium, containing insulin, dexamethasone, indomethacin and IBMX, for 15 days alongside the murine myoblast cell line C2C12, and a tumour cell line, LiSa-2, known to readily accumulate lipid and acquire the characteristics of fat cells. A proportion of C2C12 cells accumulated large lipid droplets in the cytoplasm as detected by staining with nile red, while Pmi28 cells appeared to undergo myogenesis and fusion, albeit at sub-optimal levels, to form myotubes. Although accumulation of lipid does not necessarily mean the cells are undergoing adipogenic differentiation at a molecular level, it is a visible characteristic of adipocytes and was used here as a marker for a cellular response to the induction medium.

Reports in the literature suggest that muscle satellite cells and derived myoblast cell lines can undergo transdifferentiation to a variety of alternative mesenchymal lineages in the presence of appropriate stimuli (Asakura et al., 2001; Shin et al., 2000). These preliminary results on the comparison of multipotency in Pmi28 and C2C12 myoblasts suggest that the Pmi28 cell line may represent a more committed myogenic lineage. Given that Pmi28 myoblasts appear to show no tendency towards transdifferentiation, and that mononucleate progeny resulting from cellularisation of myotubes by 109 retain MHC expression and thus represent a substrate even less likely to deviate from the myogenic lineage, these transdifferentiation experiments were taken no further. It is worth noting that the C2C12 cell line was used in both the original work with myoseverin (Rosania et al., 2000) and the conditional expression of Msx-1 in mammalian myotubes (Odelberg et al., 2000) in which the mononucleate progeny displayed multipotency, although the significance of that work lay not in demonstrating the reversal of lineage commitment but rather the reversal of the entire myogenic programme from the terminally differentiated state of the myotube.

The use of time-lapse microscopy has been a key element in this work and has provided a clear and effective means by which to follow mononucleates over a four-day period. The techniques employed in previous studies, imaging at less frequent timepoints as with myotubes expressing Msx-1 (Odelberg et al., 2000), or incorporation of BrdU in myoseverin treated cultures (Rosania et al., 2000), permit a degree of ambiguity which is absent in the direct application of time-lapse microscopy used here. The work described in this thesis demonstrates that 109 is able to induce cellularisation of mammalian myotubes to generate mononucleate cells, but that these cells do not proliferate and continue to express a muscle-specific marker. Results showing the retention of MHC and the absence of centrioles in these mononucleates suggest that the cells remain committed to the myogenic lineage and arrested from the cell cycle. These observations indicate that 109 is not able to set in motion the full programme of events activated in the endogenous urodele regeneration programme or in mammalian myotubes expressing Msx-1, but that it may represent part of the pathway. The role of Msx-1 in urodele regeneration, and its implications for mammalian systems, remains to be elucidated, as do the endogenous signals that lie upstream of Msx-1, but work on this protein to date

suggests it may be a key regulator of regeneration in urodeles and potentially in mammals.

Dedifferentiation of multinucleate myotubes during urodele regeneration involves a dramatic rearrangement of the cytoskeleton to bring about cellularisation accompanied by re-entry into the cell cycle in order to generate viable, proliferating mononucleate cells. This complex process requires the coordinated activation of cell cycle machinery, possibly involving reorganisation of centriolar components, depolymerisation of microtubules, cellular fission without loss of membrane integrity and down-regulation of muscle-specific proteins. How these different aspects are triggered, linked together and regulated is currently unknown but the work described here with 109 has begun to dissect the process by targeting one specific element, the reorganisation of the microtubule cytoskeleton, and asking what lies downstream of this and what are the direct consequences for the cells.

I have previously discussed the significant role of microtubules in myotube morphology and their depolymerisation as a key aspect of cellularisation, and in this chapter have looked at the consequences of cellularisation for individual mononucleate progeny. The results described here indicate not only that cell cycle re-entry is not a prerequisite for cellularisation of myotubes, but also that cellularisation does not inevitably trigger S phase re-entry. Ultimately cellularisation alone, characterised by the depolymerisation of microtubules, is not sufficient to activate a wider programme of dedifferentiation. However the mononucleates generated by the action of 109 may represent an intermediate step in dedifferentiation; terminally differentiated myotubes are multinucleate by definition and reversal of the mononucleate to multinucleate transition is a significant event in the dedifferentiation process. These mononucleates may provide a useful substrate for the future investigation and elucidation of endogenous, or synthetic, molecules which can reverse other aspects of differentiation, such as cell cycle withdrawal, in mammalian myotubes.

Chapter 6

Summary

Skeletal muscle is an excellent system for the study of events surrounding the differentiation process as it provides two useful indices of differentiation; cell cycle withdrawal and the mononucleate to multinucleate transition. During urodele limb regeneration, post-mitotic, multinucleate myofibres undergo dedifferentiation to generate proliferating, mononucleate cells which contribute to the blastema, reversing both indices of differentiation in the process. The work described here has been concerned with this process of dedifferentiation, and in particular with the reversal of the mononucleate to multinucleate transition, referred to as cellularisation, and has sought to assess the possibility of inducing this process in mammalian cells. This thesis has investigated the mechanism of action of myoseverin, a small purine-based molecule isolated from a combinatorial chemical library, and reported to bring about cellularisation of mammalian myotubes with an accompanying gene expression profile indicating that a programme had been activated in mammalian cells, characteristic of that involved during urodele regeneration.

A major aspect of this thesis, has been the demonstration of cellularisation at a single cell level. Cellularisation is a poorly understood process but represents reversal of a significant aspect of differentiation in skeletal muscle, and is essential for the generation of viable mononucleate cells. Previous reports of cellularisation, in both urodele and mammalian systems, have failed to offer such a convincing display of cellularisation and have lacked the resolution, and depth of description, made available here. Through the use of time-lapse microscopy I have presented evidence that viable mononucleate cells can be formed from a multinucleate myotube, and that this process can be induced in mammalian cells by myoseverin and its derivatives.

That tubulin was the only known target of myoseverin, and compound 109, was established previously, but the work in this thesis has firmly recognised a role for microtubules in the cellularisation process. The comparison of 109 with the microtubule-depolymerising agent nocodazole, demonstrated that nocodazole was also able to bring about the cellularisation of mammalian myotubes, and suggested a common mechanism of action for the two molecules. The finding that nocodazole was able to mimic the effects of myoseverin, without cellular toxicity, was exploited in the microarray study to distinguish the microtubule-binding effects of 109, from other possible effects; a subset

of genes altered by both molecules further supported a common mechanism. The microarray study also challenged the original claims that myoseverin is activating a regeneration-like response, involving altered expression of genes involved in wounding and a cellular response to tissue injury. No evidence for such a programme was found in my work. While cellularisation was shown to occur, reversal of other aspects of cellular differentiation was not evident at a molecular level. Collectively, this data suggests that myoseverin brings about its cellular and molecular effects through its action on microtubules and thus establishes microtubule depolymerisation as a possible mechanism for cellularisation in other systems.

This thesis has also assessed the consequences of cellularisation for mononucleate progeny. Again time-lapse microscopy was a powerful tool to demonstrate events occurring at the level of single cells, and the use of a motorised stage enabled the observation of multiple cellularisation events, incorporating the resolution of single cell analysis, at a scale to engender confidence in the results obtained, and add statistical weight to the conclusions. Mononucleate cells, resulting from cellularisation, were never seen to divide despite culture in high serum medium, leading to the conclusion that cellularisation is insufficient to stimulate reversal of other aspects of differentiation such as S phase re-entry and down-regulation of muscle-specific proteins.

The results described in this thesis have investigated the mechanism of action of myoseverin and expanded the original work carried out with the molecule. Although myoseverin is clearly capable of reversing the mononucleate to multinucleate transition which occurs during myogenic differentiation, my results are not consistent with the activation of a programme in mammalian cells characteristic of that involved in urodele regeneration. Instead, myoseverin has highlighted one aspect of the dedifferentiation process in isolation, that of cellularisation. The mechanism by which the complex events of dedifferentiation, incorporating cellularisation and cell cycle re-entry, are coordinated during urodele limb regeneration (Kumar et al., 2000; Lo et al., 1993), or in mammalian myotubes in response to Msx-1 expression (Odelberg et al., 2000) is unknown. Cellularisation itself is not enough to generate dividing mononucleates, and the absence of centrioles in the resulting mononucleates suggests that there are multiple aspects to the

process, only by understanding these different aspects are we likely to determine the cellular and molecular regulation of such a complex process.

While the results in this thesis do not support a gene expression profile such as that shown in the fibroblast response to serum (Iyer et al., 1999), or during *in vivo* muscle regeneration (Goetsch et al., 2003; Porter et al., 2002; Summan et al., 2003), involving genes with a role in inflammation, injury response and wounding, this is perhaps consistent with the limited reversal of differentiation occurring in response to 109. The examination of the transcriptional response of urodele myofibres during blastema formation remains impractical on the scale of microarray analysis due to the scarcity of genetic information, although genome sequencing will eventually make this possible. However, the emergence of new models of dedifferentiation and regeneration, in other organisms and cell types, such as in *Drosophila*, where differentiating germline cells can reportedly undergo dedifferentiation to repopulate the stem cell niche (Brawley and Matunis, 2004; Kai and Spradling, 2004), opens the possibility of examining and manipulating the genes involved in such processes, in a more genetically tractable system. Work on the study of S phase re-entry in both newt and mouse myotubes, and, in one study, hybrid myotubes of the two (Velloso et al., 2001), suggest that the molecules and pathways which mediate cell cycle withdrawal during differentiation are conserved in urodeles and mammals. The application of newt blastema extract to mammalian cells (McGann et al., 2001), and studies on the possible role of Msx-1 in cellularisation of urodele (Kumar et al., 2004) and mammalian (Odelberg et al., 2000) myotubes also generate optimism that activation of regeneration-like responses in mammalian cells is a plausible and achievable goal.

The discovery of myoseverin has also provided an excellent example of the value of chemical biology in characterising biological processes and identifying cellular targets. This is a growing field and one which is likely to continue to make a significant impact in many aspects of scientific research. Investigators are beginning to use this approach to identify small molecules which can alter cell fate, and may be invaluable, not only as therapeutics, but to our understanding of differentiation, cell cycle control and morphology. Reversine is another purine-based molecule reported to affect myogenic cells. In this case, the compound apparently reverses commitment to the muscle lineage,

enabling mononucleate myoblasts to revert to a pluripotent state in which they can be induced to differentiate into a range of different lineages under the appropriate culture conditions (Chen et al., 2004). The relevance of this molecule to the study of regeneration and cellularisation remains to be determined. Myoseverin has successfully reversed one aspect of the differentiated state, the mononucleate to multinucleate transition, but is unable to regulate other changes to the cell; the mononucleates remain in cell cycle arrest, continue to express MHC and do not possess centrosomes. It will be interesting to determine whether reversine can affect any of these features of the mononucleate progeny of myoseverin-treated myotubes, or indeed whether a screen using these mononucleate cells as a substrate could identify an alternative molecule capable of inducing S phase re-entry, or down-regulation of muscle-specific markers in these cells.

Given that differentiation is a carefully regulated process comprising distinct and separable steps, it is reasonable to suppose that dedifferentiation may proceed through a number of steps, and that a multi-step screening process could be designed to further dissect the events of dedifferentiation. As libraries containing tens of thousands of compounds are synthesised, and the technology to screen them in a matter or days becomes available, a critical aspect is the design of assays to enable the extraction of meaningful information from experiments conducted on such a vast scale. Myotube cultures, first treated with myoseverin to induce cellularisation, could then be screened for compounds which activate cell cycle re-entry. Alternatively, the myotubes could require pre-treatment in order to enable the progeny of subsequent myoseverin-induced cellularisation, to undergo proliferation. A compound identified such a screen could perhaps regenerate or reorganise the centrosomal machinery, ensuring that resulting mononucleate progeny each received the requisite two centrioles; or maybe target and inhibit CKIs or other regulators of the cell cycle which maintain the post-mitotic arrest.

Although the significance of centriole loss in myotubes and its consequences for regeneration in both urodeles and mammals is currently unknown, screens could be designed to further probe these questions. The removal of centrosomes from a variety of cell types, using lasers or microsurgery, results in cells which can complete mitosis but become blocked in G1 of the subsequent cell cycle, unable to enter S phase. Screening of these cells for compounds which enable the cells to progress through the cell cycle,

either by generating new centrosomes, or bypassing the requirement for them, could indicate a mechanism by which mononucleate cells generated by cellularisation of myotubes may synthesise DNA and proliferate. Similarly, myotubes, or mononucleate progeny generated by myoseverin, could be screened for compounds which can reconstitute the radial microtubule array, required for cell division, from the linear arrangement which forms during myogenesis. An assay could be designed to detect molecules which induce microtubule re-growth, following a brief period of depolymerisation, from a single aster, rather than from around the nucleus as previously observed in myotubes (Musa et al., 2003; Tassin et al., 1985). Such reorganisation of the cell's primary MTOC could be considered to constitute reversal of another aspect of the differentiation process which occurs during myogenesis. The possibilities for chemical biology are extensive, if Msx-1 is truly a master regulator of regeneration and dedifferentiation then a compound acting up-stream of the homeobox gene could conceivably activate the entire process in a manner similar to that occurring in the newt blastema. However, the potential for discovery of small molecules which can manipulate the process in a step-wise manner is an attractive possibility for those seeking to dissect and understand the coordination and interdependency of the individual aspects of dedifferentiation.

Finally, the rationale for continuing this work and expanding our understanding of the processes involved in urodele regeneration, remains more than the desire for knowledge and the elucidation of the molecular basis of a complex phenomenon. As we discover more about plasticity of the differentiated state inherent in some mammalian cells, and the potential of embryonic, and tissue-specific stem cells in regenerative medicine, the need to understand the differentiated state and manipulate it to provide therapeutics has become a driving force for such research. Already, cell transplantation is used to treat certain diseases. Transplantation of islet cells for diabetes sufferers (Swift et al., 1998; White et al., 2001), hepatocytes as an alternative to whole organ transplant in the case of inherited metabolic diseases (Ostrowska et al., 1999), transplant of dopaminergic neurons into Parkinsons disease patients (Brundin et al., 2000), and myoblasts into DMD sufferers (Partridge et al., 1989; Skuk and Tremblay, 2000) are all current realities, but all suffer from low success rates and common problems of host rejection and issues surrounding extended treatment with immunosuppressive drugs, as well as the shortage of donor tissue. Bone marrow-derived cells have been suggested as therapeutics in some

conditions (Dahlke et al., 2004; Horwitz et al., 1999) but the contribution of these cells to the target tissues is unclear. It would be of enormous benefit to be able to manipulate cells in culture to enhance survival and ensure a ready supply of donor material.

Treatment of myoblasts in culture with tubulyzine, a myoseverin-related compound, has been reported to enhance the success of myoblasts transplanted into patients with DMD (El Fahime et al., 2003), while the differentiation, *in vitro*, of CNS precursors, or cells of a human embryonic carcinoma stem cell line, into dopaminergic neurons, prior to transplantation into patients with Parkinsons disease has been reported (Iacovitti et al., 2001; Sanchez-Pernaute et al., 2001). One problem is generating a well-characterised population with a high proportion of differentiated cells. The discovery of molecules and culture conditions, which can efficiently direct the differentiation of an embryonic stem cell line *in vitro*, is a major task. In addition, stem cells, or even differentiated cell types, from the patient's own tissue would provide the ideal therapeutic if such cells could be expanded and manipulated effectively in culture.

The therapeutic potential of being able to direct the fate of cells *in vitro*, or ultimately *in vivo*, is a motivating prospect. As scientists continue to work towards these goals, the benefits of understanding the cellular and molecular basis of differentiation in many different cell types, including muscle, will be of great significance, not only in regenerative medicine, but also in the wider context of scientific research. The study of urodele amphibians which undergo dedifferentiation naturally following limb amputation, is a valuable tool in seeking to understand the differentiated state and evidence suggests that many, if not all, of the intracellular components required to undergo such dedifferentiation are conserved in mammals. The use of different systems to study dedifferentiation and regeneration will hopefully enhance our understanding of these processes. Myoseverin was originally discovered by investigators looking for dedifferentiation of the type observed in urodeles, and now the further study of myoseverin, described in this thesis, has hopefully enhanced our understanding of cellularisation in a way which may then impact back on the field of urodele regeneration, and be utilised in future studies of newt and axolotl limb regeneration. In this way, reciprocal exchange of information between different systems can help to characterise, and enhance our overall understanding of a complex process such as dedifferentiation.

Chapter 7

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